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Phosphoinositide 3 Kinase Mediates Toll-Like Receptor 4-Induced Activation of NF-κB in Endothelial Cells

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Many of the proinflammatory effects of gram-negative bacteria are elicited by the interaction of bacterial lipopolysaccharide (LPS) with Toll-like receptor 4 (TLR4) expressed on host cells. TLR4 signaling leads to activation of NF-κB and transcription of many genes involved in the inflammatory response. In this study, we examined the signaling pathways involved in NF-κB activation by TLR4 signaling in human microvascular endothelial cells. Akt is a major downstream target of phosphoinositide 3 kinase (PI3-kinase), and PI3-kinase activation is necessary and sufficient for Akt phosphorylation. Consequently, Akt kinase activation was used as a measure of PI3-kinase activity. In a stable transfection system, dominant-negative mutants of myeloid differentiation factor 88 (MyD88) and interleukin-1 (IL-1) receptor-associated kinase 1 (IRAK-1) (MyD88-TIR and IRAK-DD, respectively) blocked Akt kinase activity in response to LPS and IL-1β. A dominant-negative mutant (Mal-P/H) of MyD88 adapter-like protein (Mal), a protein with homology to MyD88, failed to inhibit LPS- or IL-1β-induced Akt activity. Moreover, a dominant-negative mutant of p85 (p85-DN) inhibited the NF-κB luciferase activity, IL-6 production, and IκBα degradation elicited by LPS and IL-1β but not that stimulated by tumor necrosis factor alpha. The dominant-negative mutant of Akt partially inhibited the NF-κB luciferase activity evoked by LPS and IL-1β. However, expression of a constitutively activated Akt failed to induce NF-κB luciferase activity. These findings indicate that TLR4- and IL-1R-induced PI3-kinase activity is mediated by the adapter proteins MyD88 and IRAK-1 but not Mal. Further, these studies suggest that PI3-kinase is an important mediator of LPS and IL-1β signaling leading to NF-κB activation in endothelial cells and that Akt is necessary but not sufficient for NF-κB activation by TLR4.

Sepsis and sepsis syndromes are life-threatening conditions and are leading causes of death in intensive care units. Sepsis is most commonly caused by gram-negative bacteria (10). Lipopolysaccharide (LPS) is an important component of the bacterial wall that contributes to the pathogenesis of sepsis. Endothelial cells (EC) are a major target of LPS and the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) (9). EC activation plays a critical role in the development of the inflammatory response in sepsis. LPS, IL-1β, and TNF-α all directly activate EC and elicit an array of EC responses, including the up-regulation of cytokines, chemokines, adhesion molecules, and procoagulant proteins (10). Most of these proinflammatory responses are mediated by the transcription factor NF-κB, which is also important for cell survival (15).

The cellular receptor transducing the LPS signal has been identified as Toll-like receptor 4 (TLR4) (13, 22), a member of the larger family of TLRs. The TLRs are an evolutionarily conserved signaling pathway involved in the induction of the innate immune response (28). LPS binds to LPS binding protein in the circulation, and this complex is recognized by soluble CD14. The LPS-LPS binding protein-soluble CD14 complex is recognized by TLR4 on EC (13). The IL-1 receptor (IL-1R) shares sequence homology with the cytosolic domain of the TLR (18, 20). The homologous cytoplasmic domain of both IL-1R and TLR is referred to as the Toll/IL-1R homology (TIR) domain. Both receptors trigger similar intracellular signaling pathways, most notably via NF-κB, which result in the induction of a variety of effector genes. Both TLR4 and IL-1R recruit the intracellular protein myeloid differentiation factor 88 (MyD88) via respective TIR domain interactions. These interactions result in the recruitment of IL-1R-associated kinase 1 (IRAK-1) to the receptor complex, where it interacts with TNF receptor-associated factor 6, resulting in the downstream activation of NF-κB (2).

In addition to NF-κB, another important pathway regulating immunity and inflammation is phosphoinositide 3 kinase (PI3-kinase). PI3-kinase is a ubiquitous lipid kinase that phosphorylates the 3 position of the inositol ring of inositol phosphoinositides to generate lipid messengers. It is composed of a p110 catalytic subunit and a p85 regulatory subunit. In p110γ-null mice, neutrophils and macrophages migrate poorly in response to various chemokines, and the chemokine-induced oxidative burst is reduced in neutrophils (23, 29, 36). The p110γ-null mice also show a reduced inflammatory response to bacteria (23). p85-null mice have impaired pre-B-cell development, reduced numbers of mature B cells in the spleen, decreased antibody production, and reduced B-cell proliferation in response to LPS (16, 38). In addition, PI3-kinase activation is involved in T-cell activation and proliferation in response to

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ligation of several surface molecules, such as the T-cell receptor CD28 and IL-2 (41).

PI3-kinase has been shown to mediate several biological effects of LPS and IL-1, including monocyte survival and monocyte proliferation (8, 19), lymphocyte and neutrophil activation (39, 42), and EC survival (30). However, it has not been determined whether the PI3-kinase activation is involved in TLR4 signaling in EC. In this study, we demonstrate that LPS or IL-1β activates PI3-kinase in EC and that MyD88 and IRAK-1 mediate this activation. We also provide evidence that PI3-kinase is necessary for NF-κB activation by LPS or IL-1β.

MATERIALS AND METHODS

Reagents. Human microvascular EC (HMEC) were provided by F. J. Candal and E. Ades (Centers for Disease Control and Prevention, Atlanta, Ga.) and T. Lawley (Emory University, Atlanta, Ga.) (1). Cell culture media and supplies were obtained from BioWhittaker (Walkersville, Md.). Recombinant human TNF-α and IL-1β were purchased from R&D Systems (Minneapolis, Minn.). LPS from *Escherichia coli* serotype 0111:B4, which was phenol extracted and further purified by ion-exchange chromatography, was purchased from Sigma Chemical Co. (St. Louis, Mo.). Anti-IκBα, anti-p85α, and glycogen synthase kinase 3α/β (GSK3α/β) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Anti-phospho-Akt, anti-phospho-GSK3α/β, anti-Akt antibodies, Akt, and c-Jun N-terminal kinase (JNK) assay kits were purchased from Cell Signaling Technology, Inc. (Beverly, Mass.). The cDNAs encoding a dominant-negative mutant p85 (p85-DN), p85ΔiSH2-N, and dominant-negative mutant Akt (Akt-AH) were kindly provided by Julian Downward (Imperial Cancer Research Fund, London, United Kingdom). The cDNAs encoding the TIR domain of MyD88 (MyD88-TIR) and the death domain of IRAK-1 (IRAK-DD) were generous gifts from Marta Muzio (Mario Negri Institute, Milan, Italy). The cDNA encoding a mutant version (Mal-P/H) of MyD88 adapter-like protein (Mal) was provided by Ruslan Medzhitov (Yale University, New Haven, Conn.).

Cell culture. HMEC were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 12 μg of bovine brain extract (Clonetics, Walkersville, Md.) per ml and maintained at 37°C in 5% CO_2 as previously described (7).

Retrovirus generation and stable infection. The cDNA encoding p85-DN, Akt-AH, MyD88-TIR, IRAK-DD, or Mal-P/H was cloned into the bicistronic retroviral expression vector pBMN-IRES-EGFP (kindly provided by Garry. P. Nolan, Stanford University, Stanford, Calif.) (27). Retrovirus was generated from the Phoenix amphotropic packaging cell line (American Type Culture Collection, Manassas, Va.) transfected with each individual construct. Retroviral supernatants were collected 48 h after transfection. For infection, HMEC were incubated with retroviral supernatant for 10 h, after which the retroviral supernatant was replaced with growth medium. Two days later, cells were analyzed and sorted on the basis of enhanced green fluorescent protein (EGFP) expression, using a FACVantage SE cell sorter (Becton Dickinson Corp., Franklin Lakes, N.J.). The EGFP-positive cells were cultured for subsequent experiments. The stably transduced cells used for experiments were pooled populations.

Adenovirus infection. HMEC were seeded at 2×10^5 cells/well in six-well plates. On the next day, the cells were incubated at a multiplicity of infection (MOI) of 500 with adenovirus containing either control EGFP or constitutively activated Akt (Akt-CA) cDNA in complete medium. After a 2-h incubation at 37°C, fresh medium was added, and the cells were incubated at 37°C with 5% CO_2 for 16 to 18 h.

Luciferase reporter activity assay. The recombinant adenovirus luciferase reporter construct was a gift from Jim Kelly (ZymoGenetics, Inc., Seattle, Wash.), and was described previously (7). The luciferase activity was determined by using the luciferase assay kit and TopCount luminescence counter (both from Packard Instrument Co., Meriden, Conn.). Subconfluent HMEC in microtiter wells were incubated with adenovirus at an MOI of 1,000 in serum-free medium for 16 h. After 5 h of incubation with TNF-α, LPS, or IL-1β in assay medium (Ham's F-12 medium supplemented with 20 mM HEPES and 0.5% bovine serum albumin), 100 μl of reconstituted substrate per well was added to the test plate, and the plate was sealed and mixed gently in dark for 10 min. The luminescence was measured on the TopCount counter at 22°C in single-photon counting mode. NF-κB activity was reported relative to that in simultaneous medium controls.

Immunoblot analysis. After experimental treatment, the confluent HMEC monolayers were washed with ice-cold phosphate-buffered saline, lysed with ice-cold radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, protease inhibitor mixture tablet [Roche Molecular Biochemicals], 1 μg of pepstatin per ml, 1 μg of type I DNase per ml, 1 mM vanadate, 50 mM NaF). The protein was collected by microcentrifugation at 16,000 × *g* for 15 min at 4°C. The supernatants were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 4 to 20% Tris-glycine gradient gel (Invitrogen Inc., Carlsbad, Calif.) and transferred to a nitrocellulose membrane. The blots were blocked overnight with 5% skim milk in TBST (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween 20) and then incubated with primary antibodies at a 1:1,000 dilution for 1 h. The blots were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin. Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, N.J.), and exposed to Kodak X-Omat Film.

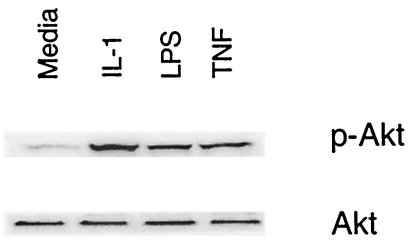

FIG. 1. Activation of PI3-kinase/Akt by IL-1β, LPS, and TNF-α. HMEC were made quiescent by incubation with EBM overnight and then treated with 10 ng of IL-1β per ml, 100 ng of LPS per ml, or 10 ng of TNF-α per ml for 20 min. Cell lysates were immunoblotted with antibody against phospho-Akt or Akt. The results shown are representative of those from three experiments.

IL-6 ELISA. HMEC were seeded into 96-well plates at 2×10^4 cells/well in 200 μl of complete medium. On the next day, the cells were incubated with various agents overnight. Supernatants were collected and analyzed with an IL-6 enzyme-linked immunosorbent assay (ELISA) kit according to the instructions of the manufacturer (Pierce Chemical Co., Rockford, Ill.).

In vitro kinase assay. Both Akt and JNK activities were measured in vitro by using kinase assay kits described by the manufacturer (Cell Signaling Technology, Beverly, Mass.). For the Akt assay, cell lysates were incubated with immobilized Akt antibody by gentle rocking for 3 h at 4°C. After washing, the pellet was resuspended in 40 μl of kinase buffer supplemented with 200 μM ATP and 1 μg of GSK3α/β fusion protein. The reaction mixture was incubated for 30 min at 30°C. The phosphorylation of GSK3α/β was measured by immunoblot assay with a phospho-GSK3α/β antibody. For JNK assay, cell lysates were incubated with 2 μg of c-Jun fusion protein beads by gentle rocking overnight at 4°C. After washing, the pellet was resuspended in 50 μl of kinase buffer supplemented with 100 μM ATP and incubated for 30 min at 30°C for the kinase reaction. The phosphorylation of c-Jun was measured by immunoblot assay with phospho-c-Jun antibody.

Statistical analysis. Data were analyzed by using Student's *t* test. Statistical differences were considered significant at a P value of <0.05. Analyses were performed with the Prism version for Macintosh (GraphPad Software, Inc., San Diego, Calif.).

RESULTS

LPS, IL-1β, and TNF-α activate PI3-kinase/Akt. The lipid products of PI3-kinase are known to target the Ser/Thr protein kinase Akt to the plasma membrane, where it is fully activated through phosphorylation on Ser473 and Thr308. The phosphorylation of Akt is PI3-kinase dependent. To assess the effects of LPS, IL-1β, and TNF-α on the PI3-kinase/Akt pathway, PI3-kinase activation was evaluated by immunoblot analysis of the phosphorylated Akt. HMEC were incubated in endothelial cell basal medium (EBM) without serum overnight and subsequently stimulated with LPS (100 ng/ml), IL-1β (10 ng/ml), or TNF-α (10 ng/ml) for 20 min, and lysates were subjected to immunoblot analysis with the phospho-Akt or non-phospho-Akt antibodies. As shown in Fig. 1, the phosphorylation of Akt increased in response to LPS, IL-1β, and

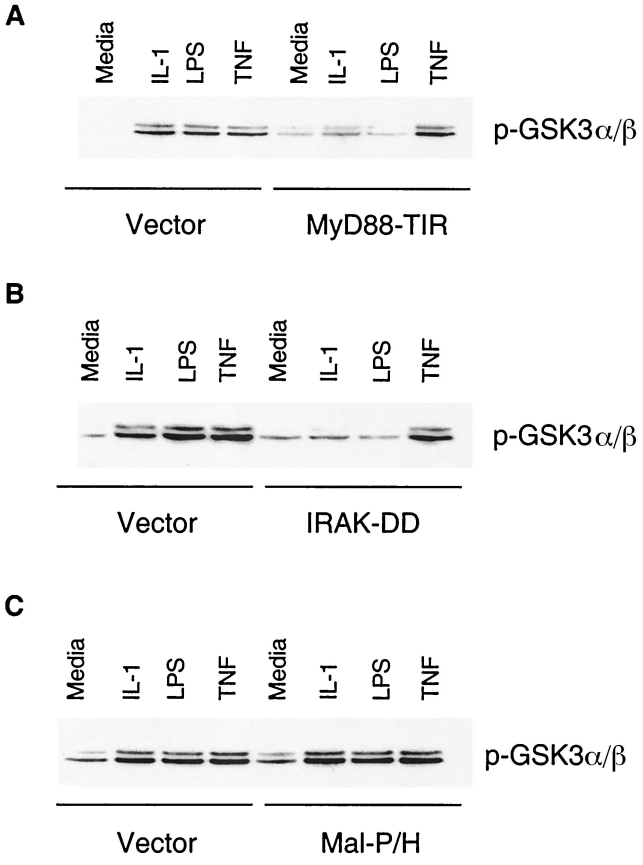

FIG. 2. Effects of different mutants on Akt kinase activity. HMEC stably transfected with MyD88-TIR (A), IRAK-DD (B), or Mal-P/H (C) were treated with 10 ng of IL-1β per ml, 100 ng of LPS per ml, or 10 ng of TNF-α per ml for 20 min. The Akt kinase activity was measured by in vitro kinase assay with GSK3α/β as a substrate. The phospho-GSK3α/β was detected by immunoblot assay. The results shown are representative of those from two (C) or three (A and B) experiments.

TNF-α. The total amount of Akt protein did not change with treatments.

Dominant-negative MyD88 blocks PI3-kinase/Akt activation in response to LPS or IL-1β. MyD88 is one of the most proximal intracellular signaling molecules involved in IL-1β- and LPS-induced NF-κB activation. MyD88 contains two distinct protein interaction domains, a C-terminal TIR domain which mediates its ability to bind to TLR and an N-terminal death domain which mediates its interaction with IRAK-1 (31). To determine whether MyD88 affects PI3-kinase activation by IL-1β or LPS, HMEC were stably transfected with a mutant version of MyD88, containing only the TIR domain, designated MyD88-TIR. A previous study by Zhang et al. showed that MyD88-TIR inhibited both IL-1β- and LPS-mediated NF-κB activation and demonstrated differences between TNF and TLR4/IL-1 signaling pathways leading to NF-κB activation (43). HMEC expressing the MyD88-TIR completely blocked Akt kinase activity stimulated by both IL-1β and LPS but not that stimulated by TNF-α (Fig. 2A). These results suggest that IL-1β- and LPS-stimulated PI3-kinase activation is mediated by MyD88 and that TNF-α-stimulated PI3-kinase activation occurs by a MyD88-independent mechanism.

Dominant-negative IRAK-1 blocks PI3-kinase activation in response to LPS or IL-1β. IRAK-1 is an adapter protein of the IL-1R and TLR signaling complex. After receptor activation, MyD88 binds to IRAK-1 through homotypic domain binding and leads to NF-κB activation by both LPS and IL-1β. In order to determine the role of IRAK-1 in PI3-kinase activation by LPS and IL-1β, HMEC were stably transfected with the gene for a truncated mutant of IRAK-1 (IRAK-DD), encoding amino acids 1 to 208 of the death domain of IRAK-1. This IRAK-DD has been shown to block NF-κB activation by both LPS and IL-1β (7, 43). As shown in Fig. 2B, IRAK-DD blocked Akt kinase activation by LPS or IL-1β. In contrast, IRAK-DD failed to block Akt activation by TNF-α. These data are consistent with the effect of MyD88-TIR on Akt activity and suggest that both MyD88 and IRAK-1 are involved in LPS- and IL-1β-stimulated PI3-kinase/Akt activation.

Mal is not required for PI3-kinase activation induced by LPS or IL-1β. MyD88 adapter-like protein (Mal) shares homology with MyD88 and can dimerize with MyD88. Similar to the case for MyD88, the C-terminal region of Mal contains a TIR domain. An important difference from MyD88 is that the N-terminal of Mal is 75 amino acids shorter and lacks a death domain. Mal and MyD88 together may be required for a rapid and optimal response to LPS. Mal is required for LPS-induced activation of NF-κB via TLR4 (14, 25). Since TLR4 is the predominant TLR on EC, we were interested in determining whether Mal was also involved in PI3-kinase activation. We and others have shown that a mutant form of Mal (Mal-P/H), in which Pro125 is mutated to histidine, blocks LPS-induced NF-κB activation (6, 14, 25). HMEC were stably transfected with Mal-P/H, and the effect of Mal-P/H on PI3-kinase in response to LPS and IL-1β was examined by in vitro Akt kinase assay. As shown in Fig. 2C, overexpression of this mutant failed to inhibit Akt activity induced by LPS and IL-1β, suggesting that Mal is not required for LPS- or IL-1β-induced PI3-kinase activation.

PI3-kinase is necessary for NF-κB activation by LPS and IL-1β but not for that by TNF-α. Whether PI3-kinase mediates NF-κB activation in EC has not been fully elucidated. To assess the role of the PI3-kinase pathway in NF-κB activation in response to these inflammatory mediators, HMEC were stably transduced by retrovirus with p85-DN. This mutant lacks amino acids 478 to 513 in the iSH2 domain and therefore does not bind to p110, the catalytic subunit of PI3-kinase. p85-DN is able to compete with native p85 for binding to essential signaling proteins, thus behaving as a dominant-negative mutant (12). The expression of p85-DN in HMEC was confirmed by immunoblotting with a p85 antibody (Fig. 3A). Figure 3B shows that the phosphorylation of Akt in LPS-, IL-1β-, or TNF-α-treated HMEC expressing p85-DN was substantially inhibited, demonstrating that p85-DN indeed functioned as a dominant-negative mutant and inhibited PI3-kinase activity.

We next examined the effect of p85-DN on NF-κB activation, which was assessed by luciferase activity as well as by IκBα degradation. HMEC expressing EGFP or p85-DN were infected with recombinant adenovirus luciferase reporter vector overnight. The luciferase assay was performed after a 5-h incubation with 100 ng of LPS per ml, 10 ng of IL-1β per ml or 10 ng of TNF-α per ml. Figure 4A shows that NF-κB luciferase activity induced by LPS or IL-1β, but not TNF-α, was substan-

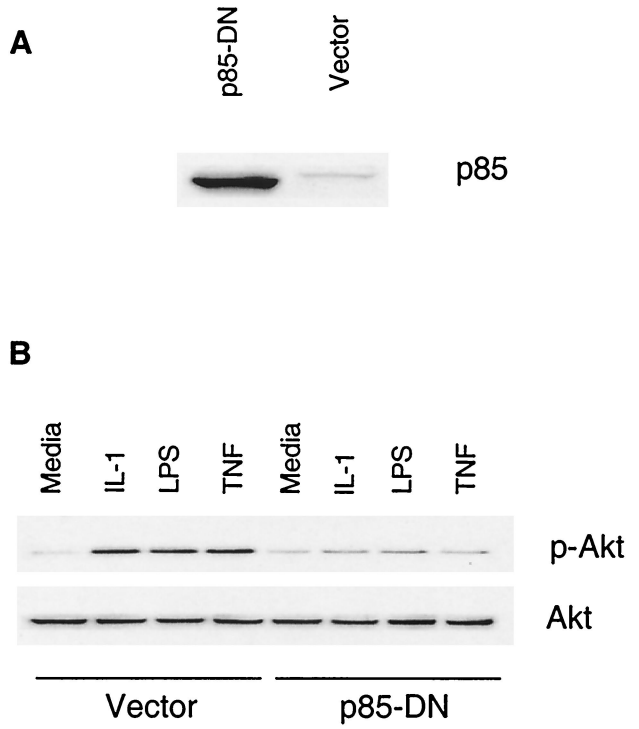

FIG. 3. p85-DN blocks the phosphorylation of Akt stimulated by IL-1β, LPS, and TNF-α. (A) HMEC were stably transfected with p85-DN or EGFP vector, and expression was confirmed by immunoblotting with antibody against p85. (B) HMEC stably transfected with p85-DN or EGFP vector were incubated in EBM overnight. The cells were then treated with 10 ng of IL-1β per ml, 10 ng of TNF-α per ml, or 100 ng of LPS per ml for 20 min. Cell lysates were immunoblotted with antibody against phospho-Akt or Akt. The results shown are representative of those from three experiments.

tially inhibited by p85-DN. These data indicate that PI3-kinase mediates LPS- and IL-1β-induced NF-κB-dependent gene expression. To determine whether the inhibitory action of p85-DN was due to its effect on IκBα degradation, the cells were treated with LPS for 60 min or with TNF-α and IL-1β for 20 min, and the cytoplasmic IκBα protein was immunoblotted with anti-IκBα antibody. IκBα was degraded within 60 min by LPS or within 20 min by IL-1β and TNF-α. Consistent with the luciferase data, p85-DN prevented IκBα protein degradation induced by LPS or IL-1β but not TNF-α (Fig. 4B). Taken together, these results suggest that activation of NF-κB by LPS and IL-1β, but not TNF-α, is PI3-kinase dependent and that inhibition of PI3-kinase prevents NF-κB activation by blocking IκBα degradation.

Inhibition of PI3-kinase blocks IL-6 release. Many inflammatory genes are regulated by NF-κB, including that for IL-6 (15). In order to confirm the results obtained with the luciferase reporter gene, we assayed for whether inhibition of PI3-kinase could block IL-6 production induced by LPS or IL-1β. HMEC expressing p85-DN or EGFP vector were incubated with LPS or IL-1β overnight, and IL-6 production was examined by ELISA. LPS- and IL-1β-induced IL-6 production was substantially inhibited by p85-DN compared with EGFP vector (Fig. 5). These results also indicate that the PI3-kinase regulates NF-κB-dependent gene expression by LPS and IL-1β.

Akt activation is not sufficient for NF-κB activation. The Ser/Thr kinase Akt has been identified as an important target of PI3-kinase, mediating a variety of PI3-kinase-dependent responses. To examine the contribution of Akt in PI3-kinase-mediated activation of NF-κB induced by LPS and IL-1β, we generated HMEC stably expressing a dominant-negative mutant Akt (Akt-AH). Akt-AH is a deletion mutant of Akt with amino acids 1 to 147 of the AH domain without the catalytic domain. AH is the domain that mediates protein-protein interaction and the formation of Akt protein complexes. Therefore, Akt-AH is able to compete with native Akt for binding to essential signaling proteins and behaves as a dominant-negative mutant (11, 21). Stable transfection of Akt-AH was confirmed by immunoblotting with Akt antibody (Fig. 6A), which recognizes the AH domain of Akt. Akt-AH inhibited NF-κB activation induced by LPS or

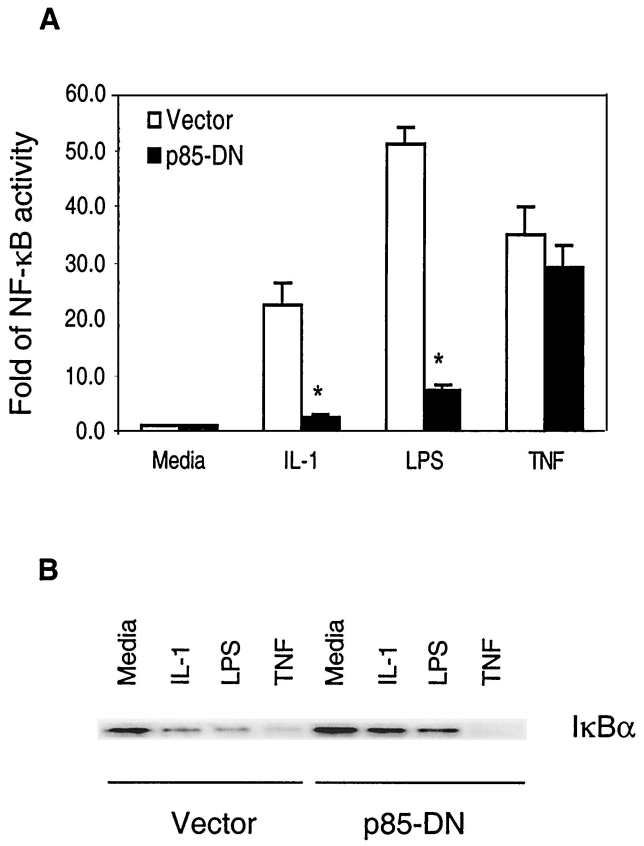

FIG. 4. p85-DN inhibits NF-κB activation in response to IL-1β and LPS but not TNF-α. (A) HMEC stably transfected with p85-DN or EGFP vector were infected with NF-κB luciferase reporter vector overnight. After 5 h of incubation with 10 ng of TNF-α per ml, 10 ng of IL-1β per ml, or 100 ng of LPS per ml, luciferase activity was measured as described in Materials and Methods. Values represent the fold increase compared with an unstimulated control and are the means ± standard deviations from five wells in a single experiment. The results shown are representative of those from three experiments. *, significant decrease compared with HMEC expressing EGFP vector ($P < 0.01$). (B) HMEC stably transfected with p85-DN or EGFP vector were treated with 10 ng of TNF-α per ml or 10 ng of IL-1β per ml for 20 min or with 100 ng of LPS per ml for 60 min. Cell lysates were immunoblotted with antibody against IκBα. The results shown are representative of those from three experiments.

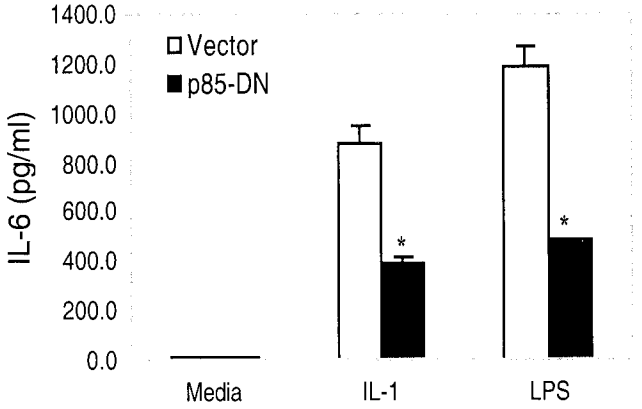

FIG. 5. p85-DN blocks IL-6 production stimulated by IL-1β and LPS. HMEC stably transfected with p85-DN or EGFP vector were treated with 10 ng of IL-1β per ml or 100 ng of LPS per ml overnight, and the production of IL-6 was measured by ELISA. Values represent the means ± standard deviations from three wells in a single experiment. The results shown are representative of those from three experiments. *, significant decrease compared with HMEC expressing EGFP vector ($P < 0.01$).

IL-1β by 50%. This inhibitory effect was significant compared with vector control cells (Fig. 6B).

In order to confirm the specificity of p85-DN and Akt-AH for PI3-kinase signaling, JNK activation by LPS and IL-1 was determined. As shown in Fig. 6C, neither LPS- nor IL-1-induced JNK activation was affected by either p85-DN or Akt-AH.

Myristylated Akt has been widely used as a constitutively activated form of Akt (Akt-CA) (35). We next examined the effect of overexpression of Akt-CA on the activation of NF-κB. HMEC were infected with adeno-Akt-CA at an MOI of 500 for 2 h, and then the infected cells were cultured for an additional 24 h. The activation of Akt was confirmed by immunoblot analysis of the phosphorylated GSK3α/β, a physiologic substrate of Akt. Figure 7A shows that expression of Akt-CA increased the phosphorylation of GSK3α/β, indicating that Akt-CA functions as a constitutively activated mutant. However, overexpression of Akt-CA alone did not induce NF-κB luciferase activity, nor did it enhance LPS- or IL-1β-induced luciferase activity (Fig. 7B). Together with the partial inhibition of NF-κB activation by Akt-AH, these results suggest that Akt activation is involved in, but not sufficient for, LPS- and IL-1β-induced NF-κB activation.

DISCUSSION

LPS-initiated TLR signaling is important for innate immunity and inflammation. EC are a major target of LPS as well as LPS-induced cytokines, such as IL-1β and TNF-α. Many EC responses to LPS, IL-1β, and TNF-α are mediated by activation of NF-κB, which is a crucial pathway for the inflammatory response. The PI3-kinase pathway is also involved in adaptive and innate immunity (23, 38, 41) and participates in a variety of biological responses to LPS (19, 24, 37, 40). Therefore, we investigated the role of PI3-kinase in TLR signaling. Here, we demonstrate for the first time that LPS activates PI3-kinase in EC and that LPS-induced activation of PI3-kinase is required for NF-κB activation. We further show that the TLR adapter proteins MyD88 and IRAK-1 mediate PI3-kinase activation by both LPS and IL-1β.

In HMEC, LPS or IL-1β, but not TNF-α, induced NF-κB activation in a PI3-kinase-dependent manner. Inhibition of PI3-kinase by p85-DN almost completely blocked NF-κB-de-

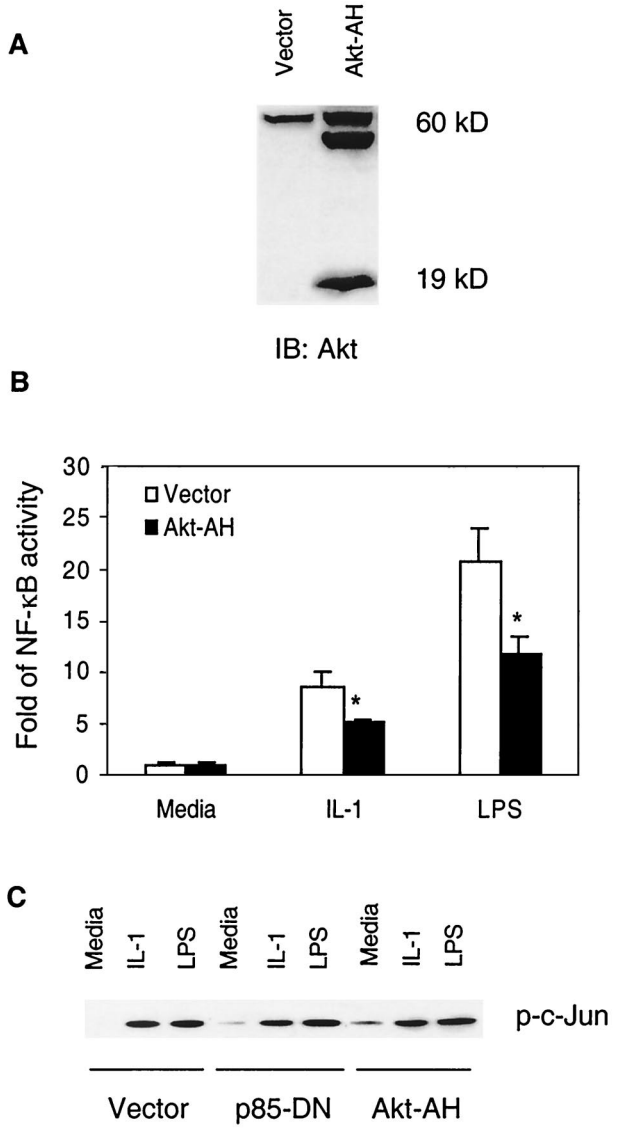

FIG. 6. Akt-AH partially inhibits NF-κB activation stimulated by IL-1β and LPS. (A) HMEC were stably transfected with Akt-AH or EGFP vector, and expression was confirmed by immunoblotting (IB) with an antibody recognizing the AH domain of Akt. (B) HMEC stably transfected with Akt-AH or EGFP vector were infected with NF-κB luciferase reporter vector overnight. After 5 h of incubation with 10 ng of IL-1β per ml or 100 ng of LPS per ml, luciferase activity was measured as described in Materials and Methods. Values represent the fold increase compared with an unstimulated control and are the means ± standard deviations from five wells in a single experiment. The results shown are representative of those from three experiments. *, significant decrease compared with HMEC expressing EGFP vector ($P < 0.01$). (C) HMEC stably transfected with GFP, p85-DN, or Akt-AH were treated with 100 ng of LPS per ml for 60 min or with 10 ng of IL-1β per ml for 15 min. The JNK activity was measured by in vitro kinase assay with c-Jun fusion protein as a substrate. The phospho-c-Jun was detected by immunoblot assay. The results shown are representative of those from two experiments.

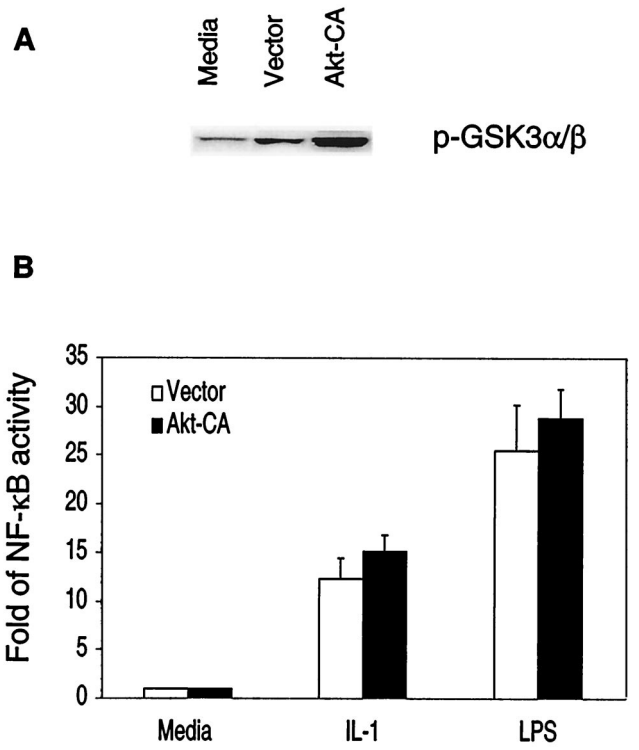

FIG. 7. Overexpression of constitutively activated Akt (Akt-CA) alone does not activate NF-κB. (A) Phosphorylation of GSK3α/β by Akt-CA. HMEC were infected with adeno-Akt-CA at an MOI of 500 for 2 h, and then the cells were cultured overnight. Following 24 h of serum deprivation, cell lysates were immunoblotted with antibody against phospho-GSK3α/β. The results shown are representative of those from two experiments. (B) HMEC were infected with adeno-Akt-CA at an MOI of 500 for 2 h, and after 24 h of incubation with growth medium, the cells were infected with NF-κB luciferase reporter vector overnight. Luciferase activity was measured after treatment with 10 ng of IL-1β per ml or 100 ng of LPS per ml for 5 h. Values represent the fold increase compared with an unstimulated control and are the means ± standard deviations from five wells in a single experiment. The results shown are representative of those from two experiments.

pendent luciferase reporter gene expression as well as IκBα degradation induced by LPS or IL-1β, but not TNF-α. The expression of p85-DN also substantially inhibited IL-6 release induced by LPS and IL-1β, which is consistent with a role of PI3-kinase in NF-κB activation by these stimuli. The signaling pathway leading to NF-κB activation initiated by LPS or IL-1β is different from that induced by TNF-α, which further supports our finding regarding the differential role of PI3-kinase in the regulation of NF-κB. In our study, p85-DN blocked IκBα degradation as well as NF-κB-dependent transcription, suggesting that PI3-kinase acts upstream of IκBα.

One well-established target of PI3-kinase is the Ser/Thr protein kinase Akt. Activation of Akt is PI3-kinase mediated (3). Previous studies showed that Akt is necessary and sufficient for NF-κB activation by platelet-derived growth factor and TNF-α in NIH 3T3 and 293 cells (33, 35). However, in HMEC, the Akt-AH construct only partially inhibited NF-κB activation induced by LPS and IL-1β, suggesting that other pathways, such as protein kinase C and p70S6-kinase, may be involved in PI3-kinase-mediated activation of NF-κB. Overexpression of Akt-CA alone did not activate NF-κB luciferase (Fig. 7). Similarly, others reported that overexpression of a constitutively activated p110 catalytic subunit of PI3-kinase was insufficient to activate NF-κB-dependent gene expression (34). These results suggest that the PI3-kinase/Akt pathway is necessary but not sufficient for NF-κB activation in HMEC.

We sought next to identify some of the signaling components involved in TLR4- and IL-1R-induced activation PI3-kinase. It is known that a tyrosine residue in the PI3-kinase binding motif (YXXM) can be phosphorylated by receptor and nonreceptor tyrosine kinases (5, 17). A previous study showed that stimulation of TLR2 by *Staphylococcus aureus* in a monocytic cell line caused the recruitment of PI3-kinase to the TLR2 cytosolic domain and that tyrosine phosphorylation of TLR2 was required for assembly of a multiprotein complex (4). However, TLR4, the predominant TLR in EC and the only EC TLR reported to bind LPS, does not contain a PI3-kinase binding motif (YXXM) in the cytosolic domain. Therefore, it is unknown whether PI3-kinase is recruited directly to TLR4 in EC.

We showed that expression of the dominant-negative protein MyD88-TIR blocked PI3-kinase activation by LPS or IL-1β, suggesting that activation of PI3-kinase by LPS or IL-1β is MyD88 dependent. Since MyD88 interacts with TLR through the TIR domain, MyD88-TIR may bind to the receptor and interfere with the binding of endogenous MyD88 to TLR. MyD88 binds to IRAK-1 through respective death domain interactions. The role of IRAK-1 in LPS- or IL-1β-stimulated PI3-kinase activation was examined by expression of the death domain of IRAK-1, IRAK-DD. Expression of IRAK-DD blocked PI3-kinase/Akt activation by LPS and IL-1β. Similar to the case for MyD88, IRAK-DD failed to block PI3-kinase/Akt activation by TNF-α, further confirming the specific effects of these mutant proteins. A recent study by Ojaniemi et al. (32) reported that PI3-kinase is involved in TLR4 signaling in a murine macrophage cell line. Consistent with our results in human EC, they found that LPS-induced NF-κB activation was mediated by PI3-kinase and that LPS induced the interaction of PI3-kinase with MyD88 (32).

Studies with MyD88-null mice suggested that there is a MyD88-independent pathway mediating NF-κB, JNK, and p38 activation during TLR signaling (26). Further studies by two groups independently identified a homologue of MyD88, Mal (14) or Toll–IL-1 receptor domain-containing adapter protein (25), which mediates MyD88-independent signaling. Mal has a TIR domain but lacks a death domain in its N terminus. Mal is a signaling component specific to the TLR4 pathway. Mal and MyD88 together may be required for a rapid and optimal response to LPS. Since TLR4 is predominant in EC, we examined the involvement of Mal in PI3-kinase activation. Overexpression of a mutant form of Mal, Mal-P/H, failed to inhibit PI3-kinase/Akt activation by LPS and IL-1β, suggesting that Mal is not an adapter molecule utilized by LPS or IL-1β to induce PI3-kinase activation.

In summary, LPS, as well as the LPS-inducible cytokines IL-1β and TNF-α, activates PI3-kinase in EC. The intracellular adapters of TLR4 and IL-1R signaling, MyD88 and IRAK-1, are required for PI3-kinase activation induced by LPS and IL-1β. Mal, a MyD88 homolog, is not involved in this process. PI3-kinase activation induced by LPS or IL-1β is essential for NF-κB activation and NF-κB-dependent gene expression, and PI3-kinase acts upstream of IκBα degradation. Akt, an impor-

tant downstream target of PI3-kinase, is involved, but its activation alone is not sufficient for the NF-κB activation.

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