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Simvastatin Suppresses LPS-Induced Akt Phosphorylation in the Human Monocyte Cell Line THP-1

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Background. **Activation of the small GTPase, Rac, requires post-translational modification by isoprenylation. Statins interfere with this process by blocking the synthesis of isoprenoid intermediates. The protein kinase Akt is a multifunctional regulator of cell behavior that has been linked to Rac activation. We have shown that lipopolysaccharide (LPS) stimulation leads to Rac activation in THP-1 cells. Therefore, we hypothesized that LPS stimulation would also activate Akt, a downstream effector of Rac, and that this may be blocked by statin pretreatment.**

Materials and methods. **THP-1 cells were maintained in 1% fetal calf serum with or without 20 μM simvastatin for 24 h, followed by LPS stimulation for increasing time. Cytoskeletal changes were observed using Alexa-Phalloidin. Akt was immunoprecipitated from total cell lysate. Activated Akt was detected by immunoblotting with a phospho-Akt antibody and was quantified by image densitometry.**

Results. **LPS stimulation of THP-1 cells results in membrane ruffling and cell polarization. Furthermore, LPS increased Akt activation in THP-1 cells when compared with the nonstimulated controls. Akt phosphorylation peaked after 15 min of LPS stimulation and was suppressed by pretreatment with simvastatin.**

Conclusions. **These data demonstrate that LPS stimulation leads to increased Akt phosphorylation, which can be suppressed with simvastatin pretreatment. This suggests one possible mechanism through which simvastatin could modulate LPS-induced signaling events in monocytes to improve the host response to Gram-negative infections.**

Key Words: **LPS; Akt; Simvastatin; statin; THP-1.**

INTRODUCTION

The actin cytoskeleton mediates a variety of essential biological functions in cells. In addition to providing a structural framework around which cell shape and polarity are defined, its dynamic properties provide the driving force for cells to move and to divide. Members of the Rho family of guanine nucleotide triphosphate (GTP)-binding proteins, such as Rac, Rho, and Cdc42, control the assembly and organization of the actin cytoskeleton to influence cell adhesion and migration, to orchestrate enhanced cytokine production, and to modulate reactive oxygen intermediate production [1]. Rho GTPases act as small molecular switches that cycle between the inactive GDP-bound state and the active-GTP bound state. In response to extracellular signals, activated Rho GTPases induce coordinated changes in the organization of the actin cytoskeleton and in gene transcription to drive a large variety of biological responses [2].

Gram-negative sepsis can be complicated by systemic vascular collapse, disseminated intravascular coagulation, and vascular leak syndromes. A common prelude to these complications is cellular dysfunction that occurs as a consequence of cytoskeletal rearrangements and alterations in intracellular signaling. Evidence exists that the Gram-negative bacterial component responsible for these cellular changes is the bacterial cell envelope constituent, endotoxin or lipopolysaccharide (LPS), a complex glycolipid composed of lipid A and a polysaccharide region that can be divided into a core region and the O-specific chain [3].

LPS is a potent activator of mammalian cells that

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express the Toll-like receptor-4 (TLR4), a member of the large class of pattern recognition receptors. Human TLRs share common signaling pathways, involving MyD88 and interleukin-1 receptor-associated kinase, that lead to the activation of multiple downstream effectors. This occurs as a consequence of the cytoplasmic domain of the TLRs, which shares significant sequence homology to the interleukin-1 receptor [4]. Although progress has been made in identifying the components of the pathway that activate gene transcription, the intracellular signaling events leading to cytoskeletal reorganization are not well defined.

The serine/threonine protein kinase B(PKB)/Akt (Akt) is a multifunctional regulator of cell migration and survival. Akt is a downstream effector of Rac. In nonstimulated cells, the Akt protein exists in an unphosphorylated form in the cytoplasm. The phosphorylated, active form of Akt is recruited to the plasma membrane of stimulated cells where it requires an intact actin network for its activation. In leukocytes, Akt has been shown to accumulate specifically at the leading edge of polarized cells where it is located in membrane ruffles [5].

Our recent work has demonstrated that LPS stimulation of the human monocytic cell line THP-1 leads to increased cell migration. Furthermore, we have determined that the observed increase in cell motility correlates with the activation of Rac, a Rho GTPase family member. In the present work, we demonstrate that 1) LPS treatment increases membrane ruffling in adherent THP-1 cells; 2) LPS stimulation results in activation of Akt, a downstream effector of Rac that accumulates in membrane ruffles; and 3) LPS-mediated Akt activation can be blocked by pretreatment of cells with simvastatin, a pharmacological agent that inhibits Rac activation. These data demonstrate that LPS-mediated signaling events in monocytes can be modulated by statin treatment.

MATERIALS AND METHODS

Cell Culture

The human monocyte cell line THP-1 was grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100U/mL), streptomycin (100 mM), glucose, essential amino acids, and sodium pyruvate. Before each experiment, the THP-1 cells were maintained in serum-depleted (1% FCS) medium for 24 h.

Reagents

All standard culture reagents were obtained from JRH Bioscience. LPS was supplied from Sigma (St. Louis, MO). Simvastatin was obtained from Merck Pharmaceuticals and solubilized in dimethyl sulfoxide. Monoclonal antibodies specific to Akt and to phospho-Akt were purchased from Upstate Biotechnology Industries.

Immunoblotting and Immunoprecipitation

Stimulation of confluent cells was quenched with serum-containing medium. Cells were lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% Na-deoxycholate, 0.5 mM EDTA, 1% NP40, 1 mM phenylmethylsulfonylfluoride, 1 mM NaVO4, 50 mg/mL leupeptin, and 0.5% aprotinin. Protein concentrations were quantified by BCA Protein Assay. For immunoprecipitations, cell extracts were incubated with the anti-Akt antibody overnight at 4°C, followed by incubation for 2 h with Protein A-agarose beads. The beads were washed four times with lysis buffer and immunocomplexes were collected and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by immunoblotting with an antibody specific to either total Akt or to the phosphorylated, active form of Akt.

Simvastatin and Akt

THP-1 cells were maintained in 1% FCS with or without 20 μM simvastatin for 24 h, followed by LPS stimulation for increasing time as indicated. Akt was immunoprecipitated from total cell lysate (250 μg). Activated Akt was detected by immunoblotting with a phospho-Akt antibody. Blots from three separate experiments were scanned and their images quantified by densitometry.

Alexa Fluorescein-Labeled Phalloidin Staining

THP-1 cells were maintained in 1% FCS for 24 h. THP-1 cells (5×10^5) were allowed to adhere to glass cover slips coated with 10 mg/mL of fibronectin for 1 h followed by LPS stimulation for 30 min. The cells were fixed in 3.7% Formaldehyde in 1× PBS +0.5 mM $MgCl_2$ followed by staining with Alexa-labeled Phalloidin (488 & 568; Molecular Probes). Cells were viewed under 20× using inverted fluorescence optics. Photographic images were captured using a Spot color camera (Diagnostic Instruments) connected to a MacIntosh G4 computer equipped with IPLab image analysis software.

RESULTS

LPS Stimulation of THP-1 Cells Alters the Actin Cytoskeleton and Induces Membrane Ruffling

Previous work has demonstrated that LPS stimulation of THP-1 cells leads to the activation of Rac, a small GTP-binding protein that regulates actin dynamics [3]. Rac is responsible for a distinct pattern of actin organization leading to lamellipodia formation. Therefore, we hypothesized that LPS stimulation of THP-1 cells would produce reorganization of the cytoskeleton consistent with this process. As demonstrated in Fig. 1A, THP-1 cells adherent to fibronectin are uniformly round. The cells are symmetric and the actin is distributed primarily around the cortex of the cells. Following stimulation with LPS however, an alteration in cell shape was readily apparent. The majority of cells demonstrate evidence of membrane ruffling with lamellipodial extension indicating polarization of the cell (Fig. 1B). These findings are consistent with an LPS-induced increase in Rac activity previously demonstrated in these cells.

LPS Stimulates Akt Phosphorylation

PKB/Akt is a downstream effector of Rac GTPases that is localized, in its active form, in membrane ruf-

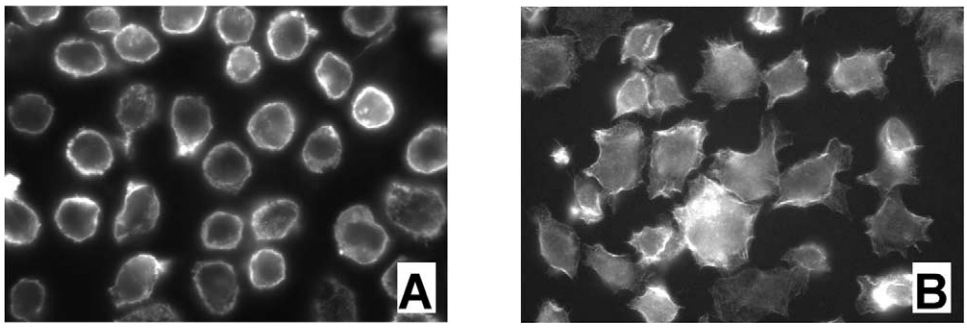

FIG. 1. LPS stimulation leads to a change in the actin cytoskeleton of THP-1 cells. THP-1 cells in 1% FCS were plated on fibronectin and allowed to adhere for 60 min. Cells remained adherent for an additional 30 min in the presence (B) or absence (A) of LPS. The cells were then fixed and stained with Alexa-labeled phalloidin to visualize F-actin.

fles. Because LPS stimulation of THP-1 cells leads both to an increase in membrane ruffling and in Rac activity, we hypothesized that LPS signaling would also result in activation of PKB/Akt. To test this idea, THP-1 cells were incubated with LPS for increasing time as indicated, and activation of Akt was assessed using phospho-specific antibodies. As demonstrated in Fig. 2A, LPS treatment leads to an increase in the phosphorylated, active form of Akt that peaks following 15 min of LPS stimulation.

Simvastatin Inhibits LPS-induced Akt phosphorylation

Simvastatin is a member of the HMG-CoA Reductase family of enzymes clinically proven to lower serum cholesterol. The pleiotropic effects of these drugs are thought to be due, in part, to their secondary effects on the synthesis of isoprenoid intermediates that are required for the membrane targeting and activation of Rho family members [6]. We have determined that pretreatment of THP-1 cells with statins, block LPS-induced Rac activation (Patel *et al*., submitted). To test whether statin pretreatment could similarly block activation of the Akt signaling pathway by LPS, cultured THP-1 cells were incubated with simvastatin prior to LPS treatment. Akt activation was assessed as described above. As demonstrated in Fig. 2A, simvastatin inhibited LPS-induced Akt phosphorylation at both 5 and 15 min. This difference was significant at the 15-min time point (Fig. 2B; $P \leq 0.05$).

DISCUSSION

In this article, we demonstrate that LPS stimulation leads to lamellipodia formation in THP-1 cells, a finding consistent with our previous demonstration of LPS-induced Rac activation in these cells. Further, we show that LPS treatment results in the activation of the Rac-effector, Akt. Finally, we demonstrate that pretreatment of THP-1 cells with simvastatin inhibits LPS-stimulated Akt phosphorylation. Taken together, these data suggest that Akt activation after LPS stimulation proceeds through a Rac-dependent signaling pathway that can be modulated by statin pretreatment. This provides a relevant biological mechanism whereby statin treatment may be used to regulate LPS-signaling in monocytes.

Members of the Rho family of GTP-binding proteins have been shown to regulate the organization of the actin cytoskeleton in response to a wide variety of stimuli. Rac activation, in particular, coordinates the formation of distinctive structures called lamellipodia, which are thought to be important for cell movement. Recent work has demonstrated that colony-stimulating factor-1 treatment of a macrophage cell line stimulated cell motility through a Rac-dependent process. In these studies, microinjection of activated Rac in macrophages directly induced lamellipodial formation and membrane ruffling, while microinjection of dominant-negative Rac blocked colony-stimulating factor-induced cell migration [1]. Our previous work has demonstrated that LPS stimulation of THP-1 cells leads to Rac activation and increased cell migration (Patel *et al*., submitted for publication). These data support the idea that Rac activation in cells of monocyte/macrophage lineage may play a central role in their ability to migrate to sites of inflammation.

The effect of LPS on the reorganization of the actin cytoskeleton has been well documented in endothelial cells but is not well described for hematopoietic cells. In endothelial cells, LPS stimulation leads to a loss of transcytoplasmic F-actin stress fibers from the central portion of the cell with a circumferential redistribution of F-actin to the cell periphery [3]. LPS-stimulated alteration in the monocyte cytoskeleton also leads to a redistribution of actin to modulate both cellular structure and subsequent cell migration. The signaling mechanism responsible for LPS's effect on the monocyte cytoskeleton has not been determined but is most likely related to LPS-induced activation of Rac [7].

Recent work has examined the effect of heat-killed *Staphylococcus aureus* (HKSA) on Rac signaling in cells that express TLR2. In this work, the authors

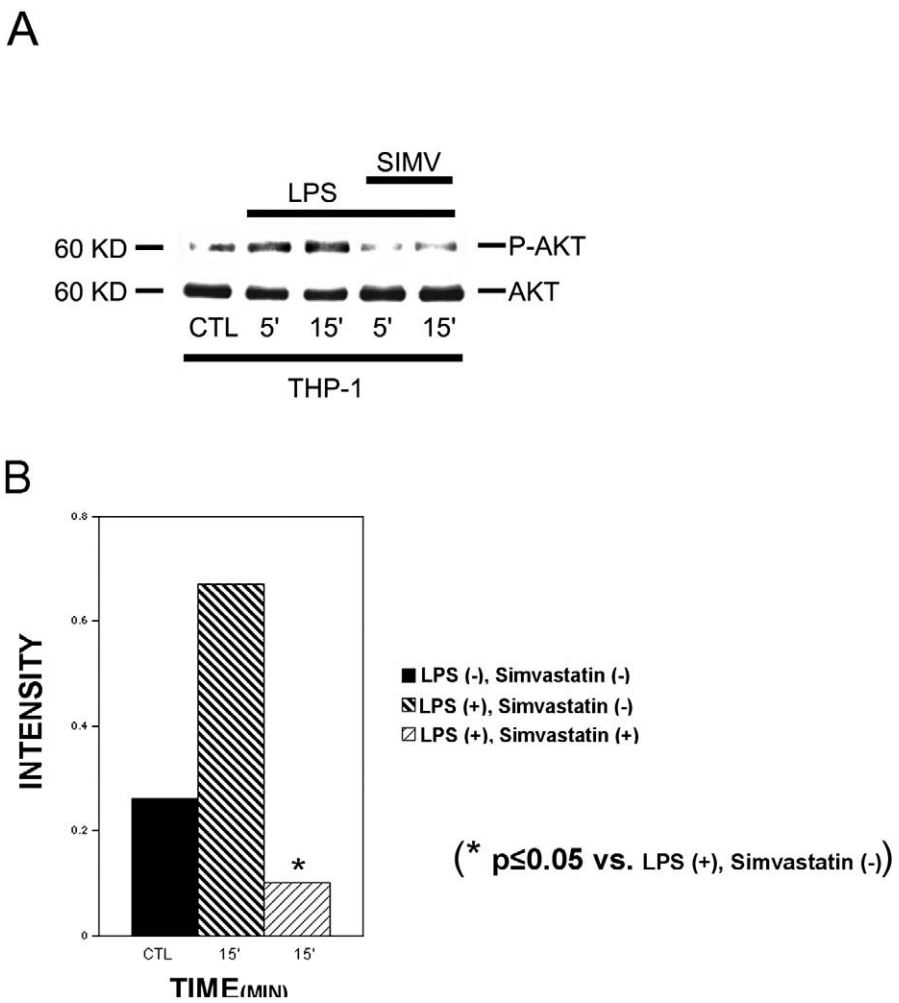

FIG. 2. A, LPS stimulation of THP-1 cells leads to Akt activation. THP-1 cells in 1% fetal calf serum were stimulated with LPS for increasing time as indicated. In some experiments, cells were pretreated for 24 h with simvastatin prior to LPS stimulation. At the indicated time, cells were lysed and subjected to immunoprecipitation with an anti-Akt antibody. Immunocomplexes were analyzed by SDS-PAGE followed by immunoblotting to detect phosphorylated or total Akt. Results are representative of three independent experiments. B, Three separate immunoblots detecting phosphorylated-Akt or total-Akt were scanned and quantitative image analysis performed using densitometry. The data are expressed as the phosphorylated-Akt/total-Akt ratio for each time point. Students' paired *t* test was used for statistical analysis. *Indicates $P \leq 0.05$ *versus* LPS (+), Simvastatin (−).

demonstrate that TLR2 stimulation by HKSA leads to a rapid and transient activation of Rac. This occurs through a recruitment of activated Rac to the cytosolic domain of TLR2. This process was dependent on both the tyrosine phosphorylation of TLR2 and the subsequent association of the TLR2 cytoplasmic domain with the p85 subunit of phosphotidylinositol-3 kinase (PI-3 kinase). As mentioned above, there is significant sequence homology between TLR2 and TLR4. The cytoplasmic domain of both receptors contains the identical consensus sequence required for tyrosine phosphorylation of the receptor. It is possible therefore, that phosphorylation of TLR4 in response to LPS may also occur and may be important to the initiation of Rac signaling events in response to LPS [4].

The HMG-CoA reductase inhibitors, or statins, are potent inhibitors of cholesterol synthesis. Large clinical trials have demonstrated that these agents reduce cholesterol and the incidence of cardiovascular diseases. Recent evidence however, suggests that the beneficial effects of statins may extend beyond their effects on serum cholesterol levels. Previous work in both endothelial and vascular smooth muscle cells has shown that statins can inhibit the synthesis of isoprenoid intermediates in the cholesterol biosynthetic pathway. These isoprenoids, geranylgeranyl and farnesyl pyrophosphates, are required for lipid modification of small GTP-binding proteins and are essential for their activation and recruitment to the plasma membrane [8].

There has been much work published recently regarding the effects of statin treatment on endothelial cells and cardiomyocytes. These effects have been

linked to changes in Rac activation. For example, recent work has shown that statins both inhibit Angiotensin II during vascular neointimal formation and reduce myocardial expression of atrial natriuretic factor and myosin light chain-2 to prevent cardiac hypertrophy in cardiomyocytes [9–11]. Our recent work has shown that statin pretreatment blocks both LPS-induced Rac activation and LPS-induced increases in THP-1 cell motility. In this article, we also have shown that statin pretreatment blocks LPS-induced Akt activation, a component of the Rac-PI-3 kinase-signaling cascade.

Akt signaling is activated by a variety of stimuli to regulate multiple critical steps in angiogenesis, including endothelial cell migration, survival and capillary structure formation [5]. Recent work has also suggested a role for Akt in the trans-activation of NF-κB in response to HKSA stimulation of cells that express TLR2. In this work, the authors demonstrate that Akt is part of a signaling cascade that originates from TLR2 and that leads to Rac and PI-3 kinase activation. TLR2-mediated Akt stimulation was shown to increase the transcriptional activity of the p65 subunit of NF-κB. This represents an important alternate pathway for NF-κB activation to the one involving MyD88, interleukin-1 receptor-associated kinase and the I-κB kinases [4]. Additionally, our work suggests that statin treatment, through its effect on both Rac and on Akt, may regulate LPS-mediated gene transcription through an effect on NF-κB.

In conclusion, we have demonstrated that LPS stimulation of the human monocytic cell line THP-1 leads to reorganization of the actin cytoskeleton and Akt activation. Further, LPS-induced activation of Akt can be blocked by statin pretreatment. These data suggest that statin treatment may have a role in modulating LPS-mediated inflammatory signaling pathways in monocytes.

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