PI3K-Akt Pathway Suppresses Coagulation and Inflammation in Endotoxemic Mice

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Objective—In endotoxemia, lipopolysaccharide (LPS) induces a systemic inflammatory response and intravascular coagulation. Monocytes orchestrate the innate immune response to LPS by expressing a variety of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), and the procoagulant molecule, tissue factor (TF). In this study, we analyzed the role of the phosphoinositide 3-kinase (PI3K)-Akt pathway in the activation of coagulation and the innate immune response in a mouse model of endotoxemia.

Methods and Results—Wortmannin and LY294002 were used to inhibit the PI3K-Akt pathway. We found that wortmannin inhibited LPS-induced Akt phosphorylation in blood cells. Inhibition of the PI3K-Akt pathway significantly increased TF mRNA expression in blood cells, TF antigen, and thrombin–antithrombin III levels in the plasma, and fibrin deposition in the liver of endotoxemic mice. Inhibition of the PI3K-Akt pathway also strongly enhanced LPS-induced cytokine expression and the levels of soluble E-selectin in the plasma, suggesting enhanced activation of both monocytes and endothelial cells. Wortmannin treatment also increased the number of macrophages in the liver and kidney of endotoxemic mice. Finally, wortmannin and LY294002 dramatically reduced the survival time of endotoxemic mice.

Conclusions—These data suggest that the PI3K-Akt pathway suppresses LPS-induced inflammation and coagulation in endotoxemic mice. (Arterioscler Thromb Vasc Biol. 2004;24:1963-1969.)

Key Words: blood coagulation ■ PI3 kinase ■ inflammation ■ endothelium ■ fibrin ■ thrombosis

Sepsis is associated with a systemic inflammatory response and intravascular coagulation, which leads to a high rate of mortality.1,2 In Gram-negative endotoxemia and sepsis, bacterial lipopolysaccharide (LPS) activates the innate immune system.1 LPS induces monocytes to express proinflammatory mediators, such as tumor necrosis factor-α (TNF-α), and the procoagulant molecule, tissue factor (TF).3–5 Excessive inflammation damages host tissue.6 LPS also activates endothelial cells, which is associated with the expression and release of E-selectin into the plasma.7–9 Induction of TF expression within the vasculature activates the coagulation protease cascade, which results in intravascular fibrin deposition.10 This may reduce blood flow and contribute to multi-organ failure. Importantly, inhibition of TF activity prevents lethality in animal models of endotoxemia and septic shock.11–13

Binding of LPS to Toll-like receptor 4 (TLR4) on the surface of monocytes leads to the recruitment of adaptor molecules, such as MyD88 and TIRAD, and the activation of various kinases, such as IRAK, IRAK-4, and TRAF-6.5,14 These kinases subsequently activate mitogen-activated protein kinase (MAPK) pathways and the 1kB kinase pathway, which leads to the activation of various transcription factors, such as AP-1 and NF-κB.15 Recent studies have shown that the phosphatidylinositol-3 kinase (PI3K) pathway regulates LPS signaling.16,17 The PI3K family is divided into 4 classes: Iα, Iβ, II, and III.18–20 Each PI3K consists of a catalytic domain and a regulatory domain. Binding of ligands to various receptors, including TLRs, activates PI3K, which then activates various downstream kinases, such as PDK-1 and Akt/PKB. The PI3K family is involved in a variety of different cell responses, such as cell survival, cell proliferation, and gene expression.18–20 The pharmacological inhibitors, wortmannin and LY294002, have been used to analyze the role of PI3K in these different cellular responses.21

We showed that the PI3K-Akt/PKB pathway negatively regulated LPS induction of TNF-α and TF expression in human monocyteic cells.16 Similarly, inhibition of the PI3K-Akt pathway enhanced LPS-induced nitric oxide synthase gene expression in murine peritoneal macrophages and TNF-α and vascular endothelial growth factor induction of TF in endothelial cells.17,22–24 Inhibition of PI3K with wortmannin or LY294002 and expression of dominant-negative Akt increased LPS activation of MAPK pathways and AP-1–dependent transcription.16 Other studies have shown that phosphorylation of Raf and extracellular signal-regulated kinase (ERK) by Akt inhibits the Raf-MEK-ERK signaling pathway.25–27 The role of the PI3K-Akt pathway in LPS

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activation of NF-κB is more controversial. We found that inhibition of PI3K enhanced LPS activation of NF-κB in monocytic cells. In contrast, other studies indicated that the PI3K pathway is required for LPS activation of NF-κB in mouse macrophage and human endothelial cells. However, in mouse macrophages, inhibition of PI3K reduced LPS-induced expression of interleukin (IL)-1α without affecting TNF-α expression.

In this study, we determined the role of the PI3K-Akt pathway in LPS-induced coagulation, inflammation, and lethality using a mouse endotoxemia model. We used wortmannin and LY294002 to inhibit PI3K. We found that inhibition of PI3K strongly enhanced LPS-induced coagulation and inflammation, and reduced the survival time of the mice. These results indicate that the PI3K-Akt pathway negatively regulates LPS-induced gene expression and lethality in vivo.

Methods

Mice

All studies were approved by The Scripps Research Institute Animal Care and Use Committee and comply with National Institutes of Health guidelines. C57BL/6J mice (8 to 12 weeks of age; Scripps Research Institute Breeding Colony, La Jolla, Calif) were used in this study. In addition, we used human chromosome vector (HCV) mice that express human TF from a human chromosome vector crossed back into the mTF/−/− background. We used a mouse model of endotoxemia that consists of intraperitoneal injection of a high dose of LPS (5 mg/kg) (Esherichia coli serotype O111:B4; Sigma Chemical Company, St Louis, Mo). Wortmannin and LY294002 were obtained from Sigma. Wortmannin (0.3 mg/kg) was injected retro-orbitally in 200 μL of ringer solution containing 10% (v/v) dimethyl sulfoxide (DMSO) (Sigma). LY294002 (40 mg/kg) was injected intraperitoneally in a total volume of 500 μL in a solution containing 10% (v/v) DMSO. DMSO was used in all mice as a vehicle control.

Measurement of Human TF Antigen in Plasma

Human TF antigen levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) (Imubind, Coagulation Diagnostica Inc).

Real-Time Polymerase Chain Reaction

Blood was collected from the inferior vena cava of anesthetized mice. Blood was mixed with sodium citrate (3.2% wt/vol) at a ratio of 10:1. Total RNA was isolated from citrated blood (0.1 mL) using 1 mL of Trizol (Invitrogen Corp, Carlsbad, Calif). Total RNA (2 μg) was incubated with DNaseI. Samples were then used to synthesize cDNA using the SuperScript First-Strand Synthesis kit (Invitrogen). To quantitate the levels of TF mRNA, PCR primers were used at a final concentration of 900 nM and the probe at a final concentration of 200 nM under standard thermocycling conditions of an ABI PRISM 7700 (Applied Biosystems, Foster City, Calif). Forward primer: mTF240F 5′-GAA ACT GGA AAA ACA AGT GCT TCT T-3′; reverse primer: mTF319R 5′-CCA GGT CAT ATC CTT CAC GAT-3′; probe: mtTF267 5′-6-FAM CCA CAG ACA CCG AGT GCG ACC trichloroacetic acid TAMRA-3′. Nucleotide numbering corresponds to NCBI accession number: NM_010171. Samples were performed in duplicate. Samples were normalized to levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as per the protocol for TaqMan Rodent GAPDH Control Reagents (Applied Biosystems).

Measurement of Thrombin–Antithrombin

Levels of thrombin–antithrombin (TAT) complex in the plasma were determined using a commercial ELISA (Enzygnost, TAT micro; Dade Behring).

Measurement of Cytokines/Chemokines and Soluble E-Selectin

Serial blood samples (0.1 mL) were collected from the retro-orbital sinus. Plasma concentrations of TNF-α, IL-6, MCP-1, KC, and soluble E-selectin were measured using commercial ELISA kits (R&D Systems).

Western Blotting

For analysis of Akt phosphorylation in blood cells, whole blood was isolated from the inferior vena cava and mixed with an equal volume of phosphate-buffered saline (pH 7.4). Samples were mixed with an equal volume of 2× sample buffer and proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to Millipore polyvinylidene fluoride membranes (Millipore). Activation of Akt was assessed using a 1:1000 dilution of an antibody that recognizes Akt phosphorylated at Ser473 (Cell Signaling, Beverly, Mass). An antibody against nonphosphorylated Akt (Cell Signaling) was used to monitor protein loading. For analysis of fibrin, total protein from the liver was isolated and quantitated as described. Proteins were separated by SDS-PAGE. The amount of cross-linked fibrin present in livers was determined by Western blotting using a mouse antihuman β-chain monoclonal antibody (59D8) (1:500) (generously provided by Dr M. Ranze, Department of Medicine, University of North Carolina, Chapel Hill) that binds to fibrin but not fibrinogen. An anticadherin antibody (Sigma Chemical) was used to monitor protein loading.

Immunohistochemistry

Mice were perfused with phosphate-buffered saline/4% paraformaldehyde. Tissues were rapidly removed, fixed in a 10% zinc formalin solution, and embedded in paraffin. Tissue sections were incubated overnight with a rabbit antimouse fibrinogen antibody (1:5000) (Dako, Carpinteria, Calif), which was detected using the Rabbit Vectastain Elite ABC kit and visualized using Vector-Red (Vector Laboratories, Burlingame, Calif). For macrophage and E-selectin staining, antigen retrieval was performed using trypsin digestion in phosphate-buffered saline with 10 mmol/L CaCl2 at 37°C for 5 minutes. Tissue sections were incubated overnight with a 1:1000 dilution of a biotinylated rabbit antibody against mouse F4/80 (Serotec, Oxford, UK), a specific macrophage marker, and chicken antibody against mouse E-selectin (R&D Systems, Minneapolis, Minn), and visualized using Vector-Red (Vector Laboratories). The number of F4/80-positive cells were counted in ≥10 high-power fields per section (3 mice per group).

Data Analysis

Cytokine/chemokine, soluble E-selectin, and TAT levels are presented as mean ± SD. Statistical analysis was performed using an unpaired Student t test. The survival of the groups was analyzed by log rank test. Differences were determined to be statistically significant at P < 0.05.

Results

Inhibition of the PI3K-Akt Pathway Reduces Mortality in a Mouse Model of Endotoxemia

We analyzed the role of the PI3K-Akt pathway in LPS-induced lethality by inhibiting PI3K with wortmannin and LY294002. A single dose of wortmannin (0.3 mg/kg) did not have any effect on the survival of endotoxemic mice (data not shown). Therefore, we used 3 doses of wortmannin (0.3 mg/kg) administered at −90, +90, and +360 minutes relative to the LPS injection.
emic mice and increased the morality compared with mice treated with LPS and vehicle (Figure 1A). Wortmannin alone did not affect the survival of mice (not shown). Similarly, LY294002 reduced the survival time of endotoxemic mice (Figure 1B). These data suggest that the PI3K-Akt pathway is protective in an endotoxemia model.

Wortmannin Inhibits LPS Activation of the PI3K-Akt Pathway in Blood Cells

We have shown that LPS stimulation of human monocytic cells activates the PI3K-Akt pathway and leads to phosphorylation of Akt. In endotoxemic mice, leukocytes are the primary source of both TNF-α and TF. Therefore, we determined if wortmannin inhibited LPS activation of the PI3K pathway in vivo by analyzing the phosphorylation of Akt in blood cells derived from endotoxemic mice. LPS activation of Akt phosphorylation at 60 minutes in blood cells was inhibited by pretreatment of mice with wortmannin (0.3 mg/kg) (Figure 2). This result indicates that administration of wortmannin abolishes LPS activation of the PI3K-Akt pathway in circulating blood cells.

Inhibition of the PI3K-Akt Pathway Enhances the Induction of TF Expression and Coagulation in Endotoxemic Mice

We have recently shown that monocytes are the primary source of TF in endotoxemic mice. In addition, LPS increases TAT levels in the circulation and fibrin deposition in various tissues in endotoxemic mice. We analyzed the effect of wortmannin on LPS-induced TF expression and coagulation by measuring TF mRNA expression in blood cells, TF antigen and TAT levels in the plasma, and fibrin deposition in the liver. Wortmannin significantly enhanced TF mRNA expression in blood cells derived from endotoxemic mice (Figure 3A). Wortmannin also strongly enhanced LPS-induced TF antigen levels and TAT levels in the plasma at 4 hours (Figure 3B and 3C). Finally, wortmannin dramatically enhanced LPS-induced fibrin deposition in the liver of endotoxemic mice (Figure 3D). Immunohistochemical analysis showed no fibrin in the liver of untreated mice (Figure IA, available online at http://atvb.ahajournals.org/). LPS treatment led to fibrin deposition in the liver (Figure IB), which was increased by treatment of the mice with wortmannin (Figure IC). Therefore, inhibition of the PI3K-Akt pathway significantly increases LPS-induced TF expression in circulating cells and in the plasma, which results in increased levels of circulating thrombin and fibrin deposition in the liver.

Inhibition of the PI3K-Akt Pathway Enhances LPS Activation of the Endothelium

In endotoxemia, LPS and cytokines induce endothelial cells to express various adhesion molecules, such as E-selectin. Some of the induced E-selectin is released into the plasma. We increased levels of soluble E-selectin in the plasma of mice treated with either LPS or LPS and wortmannin. We found that wortmannin significantly enhanced levels of soluble E-selectin in the plasma (Figure 4A). Similarly, wortmannin enhanced LPS-induced E-selectin expression on endothelial cells in the liver (Figure 4B). These results indicate that wortmannin enhances the activation of the endothelium in endotoxemic mice.

Inhibition of the PI3K-Akt Pathway Enhances LPS Induction of Cytokines and Chemokines

Intraperitoneal injection of LPS into mice induces the expression of a variety of cytokines and chemokines. We determined if inhibition of the PI3K-Akt pathway enhances LPS induction of inflammatory mediators by measuring the levels of various cytokines/chemokines in the plasma at different times. Treatment of mice with wortmannin strongly enhanced LPS induction of TNF-α, IL-6, MCP-1, and KC expression (Figure 5A to 5D). The enhancement of LPS-induced IL-6 expression by wortmannin was dose-dependent (Figure 5E). In addition, inhibition of PI3K with LY294002 enhanced LPS induction of IL-6 expression (Figure 5F). These results are consistent with the PI3K-Akt pathway suppressing LPS induction of cytokines in mice.
Inhibition of the PI3K-Akt Pathway Enhances LPS-Induced Recruitment of Blood Monocytes and/or Activation of Resident Macrophages in the Kidney and Liver

In endotoxemia, monocytes bind to activated endothelium and migrate into various tissues. We determined the effect of wortmannin on the recruitment of monocytic cells into the liver and kidney in endotoxemic mice. We observed staining of a small number of resident macrophages in the liver of untreated mice (Figure 6A). LPS increased the number of F4/80-positive cells in the liver, which was further increased by wortmannin (Figure 6B and 6C). Similar results were observed in the kidney (Figure 6E and 6F). The number of monocytes recruited into the liver and kidney of endotoxemic
mice treated with wortmannin was significantly higher than in endotoxemic mice treated with vehicle (Figure 6D and 6H). Wortmannin may also enhance LPS activation of resident macrophages in these tissues.

Discussion

Endotoxemia is associated with intravascular coagulation and a systemic inflammatory response. Monocytes are the primary source of TF in endotoxemic mice and septic baboons, although endothelial cells in the splenic microvasculature also express TF in septic baboons. In this study, we showed that inhibition of PI3K enhanced LPS-induced coagulation and inflammation, which was associated with decreased survival. We found that wortmannin inhibited LPS-induced Akt phosphorylation and increased TF mRNA expression in blood cells, presumably monocytes, derived from endotoxemic mice and TF antigen in the plasma. These results are consistent with our recent study showing that inhibition of PI3K in human monocytic cells enhances LPS-induced TF expression and other studies showing that...
wortmannin increases TF expression in endothelial cells.\textsuperscript{22–24} Endotoxemic mice treated with wortmannin also exhibited enhanced levels of TAT complex in the plasma and increased fibrin deposition in the liver. Taken together, these results indicate that the PI3K-Akt pathway suppresses LPS induction of TF expression in monocytes and limits the activation of the coagulation protease cascade in endotoxemic mice.

Inhibition of PI3K with wortmannin enhanced the LPS induction of inflammatory mediators, such as TNF-\(\alpha\), IL-6, MCP-1, and KC. In addition, wortmannin increased levels of soluble E-selectin in the plasma of endotoxemic mice and expression of E-selectin in the endothelium. Wortmannin also enhanced the recruitment of inflammatory cells into the liver and kidney of endotoxemic mice. Other in vitro studies have shown that wortmannin increases LPS induction of TNF-\(\alpha\) and inducible nitric oxide synthase in monocytes and macrophages.\textsuperscript{15,17} These data indicate that the PI3K-Akt pathway inhibits the LPS-induced expression of inflammatory mediators by monocytes and endothelial cells and suppresses the activation of the innate immune response during endotoxemia.

A recent study showed that inhibition of PI3K activity with wortmannin increased plasma cytokine levels and decreased survival in a murine model of cecal ligation and puncture-induced polymicrobial sepsis.\textsuperscript{44} Importantly, this group showed that glucan phosphate-induced PI3K activity positively correlated with increased survival in septic mice.\textsuperscript{44} The protective effect of glucan was abolished by the PI3K inhibitors, wortmannin and LY294002, suggesting that protection against mortality was mediated through PI3K.\textsuperscript{44} Glucan also provided cardioprotection in a model of myocardial ischemia-reperfusion injury.\textsuperscript{45} Similarly, an antioxidant compound, H-2693, induced Akt phosphorylation and was cardioprotective during ischemia-reperfusion in Langendorff perfused hearts.\textsuperscript{46} The protective effect of H-2693 was abolished by wortmannin.\textsuperscript{46} Finally, an inhibitor of the nuclear enzyme poly-(ADP-ribose) polymerase was shown to be protective in a model of endotoxemia by activating the PI3K-Akt pathway and decreasing the inflammatory response.\textsuperscript{47} These studies indicate that the PI3K pathway limits inflammation induced in endotoxemia, sepsis, and ischemia-reperfusion injury.

One limitation of the study was that we used pharmacological inhibition of PI3K to investigate the role of the PI3K in endotoxemia. However, wortmannin is a highly specific inhibitor of PI3K.\textsuperscript{48} In addition, the PI3K inhibitor LY294002 enhanced LPS induction of IL-6 in a similar manner to wortmannin. In a cecal ligation and puncture model, both wortmannin and LY294002 abolished the protective effect of glucan.\textsuperscript{44} In this model, wortmannin increased splenocyte apoptosis, suggesting that decreased cell survival may also contribute to the decreased survival of endotoxemic mice. These in vivo studies, together with our in vitro studies with monocytes and endothelial cells,\textsuperscript{15,22–24} strongly suggest that the PI3K pathway suppresses LPS-induced coagulation and inflammation in endotoxemic mice. We predict that inhibition of coagulation would partially reverse the detrimental effects of wortmannin.

In vitro studies indicate that major targets of the PI3K-Akt pathway are the MAPK pathways.\textsuperscript{16,22–27} The p38MAPK pathway has been shown to play a key role in the LPS induction of inflammatory mediators and coagulation in human and mouse models of endotoxemia.\textsuperscript{49–51} Inhibition of the PI3K-Akt pathway in endotoxemic mice is likely to enhance LPS activation of the p38MAPK pathway and the subsequent expression of pro-inflammatory and procoagulant molecules. The PI3K-Akt pathway may also inhibit NF-\(\kappa\)B activity.\textsuperscript{16} However, other studies indicate that PI3K is required for activation of NF-\(\kappa\)B in mouse macrophages and human endothelial cells.\textsuperscript{28,29} Mice deficient in the PI3K catalytic subunit exhibited decreased accumulation of neutrophils into the lungs of endotoxemic mice, suggesting that this member of the PI3K family plays a role in LPS-induced neutrophil activation.\textsuperscript{52} These results indicate that further studies are required to elucidate the positive and negative regulatory roles of the PI3K family in LPS signaling.

LPS activates many intracellular signaling pathways in monocyctic and endothelial cells.\textsuperscript{5,14} This study and others demonstrate that the PI3K-Akt pathway negatively regulates LPS signaling in vitro and in vivo.\textsuperscript{16,44} LPS activation of the PI3K-Akt pathway is slightly delayed relative to activation of the MAPK pathways in monocyctic cells.\textsuperscript{16} Thus, it appears that the PI3K-Akt pathway does not significantly reduce the initial LPS activation of intracellular signaling pathways but is required for the rapid shut-down of these pathways. Wortmannin enhanced LPS induction of inflammatory mediators and TF expression more dramatically at 4 hours compared with 2 hours, which suggests a defect in the shut-down mechanism. Similarly, Fukao and Koyasu\textsuperscript{53} have proposed a model in which PI3K inhibits the early phase of the innate immune response.

Sepsis and endotoxemia are associated with a systemic inflammatory response and intravascular coagulation that contribute to a high rate of mortality.\textsuperscript{1,2} Therapeutic strategies that target individual cytokines have failed.\textsuperscript{2} Our data and that of others\textsuperscript{44} indicate that drugs targeting the activation of the PI3K-Akt pathway may limit LPS-induced inflammation and coagulation and reduce the mortality associated with sepsis.

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