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*J Immunol* 2009; 182:7280-7286; doi: 10.4049/jimmunol.0801376

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Lipopoly saccharide-Induced Apoptosis in Transformed Bovine Brain Endothelial Cells and Human Dermal Microvessel Endothelial Cells: The Role of JNK

Hisae Karahashi,*† Kathrin S. Michelsen,*‡ and Moshe Arditi2*

Stimulation of transformed bovine brain endothelial cells (TBBEC) with LPS leads to apoptosis while human microvessel endothelial cells (HMEC) need the presence of cycloheximide (CHX) with LPS to induce apoptosis. To investigate the molecular mechanism of LPS-induced apoptosis in HMEC or TBBEC, we analyzed the involvement of MAPK and PI3K in TBBEC and HMEC. LPS-induced apoptosis in TBBEC was hallmark by the activation of caspase 3, caspase 6, and caspase 8 after the stimulation of LPS, followed by poly(ADP-ribose) polymerase cleavage and lactate dehydrogenase release. We also observed DNA cleavage determined by TUNEL staining in TBBEC treated with LPS. Herbimycin A, a tyrosine kinase inhibitor, and SP600125, a JNK inhibitor, suppressed the activation of caspases and lactate dehydrogenase release. Moreover, a PI3K inhibitor (LY294002) suppressed activation of caspases and combined treatment with both SP600125 and LY294002 completely inhibited the activation of caspases. These results suggest that the JNK signaling pathway through the tyrosine kinase and PI3K pathways is involved in the induction of apoptosis in LPS-treated TBBEC. On the other hand, we observed sustained JNK activation in HMEC treated with LPS and CHX, and neither ERK1/2 nor AKT were activated. The addition of SP600125 suppressed phosphorylation of JNK and the activation of caspase 3 in HMEC treated with LPS and CHX. These results suggest that JNK plays an important role in the induction of apoptosis in endothelial cells.

Lipopoly saccharide is a major component of Gram-negative bacteria and is known as a potent activator of proinflammatory responses in various types of cells (1–3). LPS can induce cell death indirectly via the secretion of TNF-α or NO in macrophages or endothelial cells (EC) (4–6). In contrast, LPS can directly induce cell death when mRNA or protein synthesis is inhibited (7). This suggests that the LPS may inhibit de novo mRNA or protein synthesis of antiapoptotic proteins and thus cause cell death of EC or macrophages.

LPS induces apoptosis in human dermal microvessel EC (HMEC) only in the presence of protein synthesis inhibitors such as cycloheximide (CHX). However, in transformed bovine brain EC (TBBEC), LPS itself induces cell death (8, 9). Mediators in both the MAPK and PI3K signaling pathways have been reported to play important roles in regulating apoptosis (10). JNK is a MAPK that responds to LPS by inducing inflammation in some cell types (11). Phosphorylation of JNK triggers UV-induced apoptosis (12) and apoptosis of EC (13). On the other hand, inhibition of JNK phosphorylation can also induce apoptosis in EC (14). These results suggest a relationship between JNK and induction of apoptosis.

To determine the molecular bases underlying differences in the response to LPS in these two types of EC (TBBEC vs HMEC), we investigated signaling by the MAPK and PI3K pathways after LPS stimulation. In this study, we show that the cell death induced by LPS in TBBEC is apoptotic and is followed by cell necrosis. Moreover, we determined that the JNK signaling pathway through tyrosine kinase and PI3K pathways plays an important role in the induction of apoptosis in TBBEC. In contrast, in HMEC, LPS does not induce phosphorylation of JNK, while CHX forced sustained phosphorylation of JNK in LPS-treated HMEC. Inhibition of JNK phosphorylation suppressed the activation of caspase 3, one feature of apoptosis. These results suggest that the JNK pathway is common in apoptosis of TBBEC and HMEC. However, in HMEC LPS alone does not induce JNK phosphorylation and subsequent apoptosis.

Materials and Methods

Cell culture

HMEC were cultured as described previously (15, 16). TBBEC were obtained from K. S. Kim (Johns Hopkins Hospital, Baltimore, MD) and isolation and purity were described extensively earlier (17) and maintained in F12/DMEM (Invitrogen) supplemented with 10% FBS, antibiotic/antimycotic, and EC growth supplement (Upstate Biotechnology).

Reagents and Abs

LY294002, SB203580, PD98059, Ac-YVAD-AMC, AC-DEVD-AMC, AC-WEHD-AMC, and AC-VEID-AMC were obtained from Calbiochem.
AC-IETD-AMC was purchased from Sigma-Aldrich. Highly purified phenol-water extracted protein-free *Escherichia coli* K235 LPS was obtained from Dr. S. N. Vogel (Uniformed Services University, Bethesda, MD). The purity of this LPS preparation has been previously demonstrated (18). AC-YVAD-CMK, AC-DEVD-CHO, and Z-Asp-CH2-DCB were from Oncogene. Anti-FLIP Ab was obtained from Upstate Biotechnology. Anti-poly-(ADP-ribose) polymerase (PARP) Ab was purchased from Santa Cruz Biotechnology. Anti-cleaved caspase 3 Ab was from Cell Signaling.

**Cytotoxicity assay**

Cells were seeded at 1.5 × 10⁵ cells/well onto 24-well plates, incubated overnight, and treated with or without LPS and incubated for various time points (A) or treated with various concentrations of LPS for 22 h (B). A LDH release assay was performed as described in Materials and Methods. The results are means ± SE for three independent experiments. *, p < 0.05. TBBEC were seeded on coverslips and incubated overnight. Cells were treated with or without LPS and incubated for 1, 2, or 4 h and stained with annexin V-fluorescein and propidium iodide (C). Representative photographs of three independent experiments are shown. ctrl, Control.

**Capase activity assay**

Cells were seeded in 10-cm dishes and incubated overnight, then treated with LPS in the presence or absence of various inhibitors for various durations. Then cells were scraped from the dishes with a cell scraper and lysed in IL-1β-converting enzyme (ICE) extraction buffer (19) (50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 20 μM cytochalasin B, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 50 μg/ml antipain, and 10 μg/ml chymopapain in 50 mM PIPES-NaOH, pH 7.0) and sonicated briefly on ice using a sonicator. Crude extracts were centrifuged at 2000 rpm for 5 min at 4°C and subsequent dilution with 100 μl of the reaction buffer (ICE standard buffer) (19), which consisted of 10% sucrose, 0.1% CHAPS, 10 mM DTT, and 0.1 mg/ml OVA in 100 mM HEPES-KOH (pH 7.5). The reaction was started by the addition of the substrate, i.e., 200 μM Ac-YVAD-AMC for caspase 1-like, 200 μM Ac-WEHD-AMC for caspase 5-like, 200 μM Ac-DEV-D-AMC for caspase 3-like, 200 μM Ac-VEID-AMC for caspase 6-like, or 200 μM Ac-IETD-AMC for caspase 8-like activity, followed by incubation at 37°C for 20 min for caspase 1-, 3-, 5-, and 6-like activity and for 10 min for caspase 3-like activity. After termination of the reaction by sudden chilling of the reaction mixture on ice, the fluorescence of the cleaved 7-amino-4-methyl-coumarine (AMC) was measured using a fluorescence microplate reader (excitation: 360 nm, emission: 460 nm). The activity of each caspase protein was calculated from a standard curve for AMC and expressed in nanomoles of AMC cleaved per min per mg cell extract protein. Time course of caspase 1-, 3-, 5-, 6-, or 8-like activity showed linearity up to 30, 15, 40, 30, and 30 min, respectively, and the dose response of cell lysates for caspase 1-, 3-, 5-, 6-, or 8-like activity was linear up to 20, 3, 20, 10, and 10 μg, respectively.

**FIGURE 1.** Time course and dose dependency of LPS-induced cytotoxicity in TBBEC. TBBEC were seeded on 24-well plates and incubated overnight. Cells were treated with or without LPS and incubated for various time points (A) or treated with various concentrations of LPS for 22 h (B). A LDH release assay was performed as described in Materials and Methods. The results are means ± SE for three independent experiments. *, p < 0.05. TBBEC were seeded on coverslips and incubated overnight. Cells were treated with or without LPS and incubated for 1, 2, or 4 h and stained with annexin V-fluorescein and propidium iodide (C). Representative photographs of three independent experiments are shown. ctrl, Control.
Detection of apoptotic cells by TUNEL and annexin V/propidium iodide staining

Cells were seeded onto glass chamber slides. Cells were treated with or without LPS for various time periods and cells were fixed with 3% formaldehyde. TUNEL staining was performed by using an In Situ Cell Death Detection, POD from Roche Diagnostics according to the manufacturer’s protocol. Stained cells were observed under the microscope and >300 cells were examined for TUNEL positivity. The results are expressed as relative percentage of TUNEL+ cells, based on the total number of cells counted. For annexin V/propidium iodide staining, cells were pretreated with DMSO or inhibitors for 1 h, followed by treatment with or without LPS for various time periods. Cells were washed with PBS and incubated with Annexin V-FLUOS labeling solution (Roche Diagnostics) and analyzed using a fluorescence microscope.

Statistics

Data are reported as the means ± SE. The statistical significance of differences between mean values was determined by Student’s t test. A value of p < 0.05 was considered significant.

Results

LPS induces cell death through apoptosis in TBBEC

After stimulation with LPS, we observed cell death of TBBEC that was characterized by shrinkage, appearance of apoptotic bodies, and nuclear condensation as early as 10 h after LPS stimulation. Next, we determined LDH release, which detects extracellular LDH after disintegration of the cell membrane. As shown in Fig. 1, LDH release increased over time in a linear fashion up to 24 h after stimulation with 100 ng/ml LPS. Without LPS, almost no cell damage was observed (Fig. 1A). LPS induced LDH release in a dose-dependent manner and saturation was observed for stimulation with >100 ng/ml LPS at 22 h of stimulation (Fig. 1B). Therefore, we used these experimental conditions (100 ng/ml LPS for 22 h in TBBEC) for all LDH release experiments.

Next, we asked whether the cell death observed in TBBEC treated with LPS was apoptosis. First, we analyzed annexin V staining in LPS-treated TBBEC as a marker for early apoptotic events. We were able to detect annexin V-positive cells as early as 1 h after LPS stimulation (Fig. 1C). Next, DNA cleavage as measured by TUNEL staining was detected as early as 4 h after LPS stimulation (Fig. 2A). Furthermore, PARP cleavage was examined because PARP is one target of activated caspases (20–23). As shown in Fig. 2B, PARP was cleaved at 8 h after the addition of LPS and cleaved PARP accumulated over time. These results suggest that LPS induces apoptosis in TBBEC.

To identify what signaling pathways are involved leading to cell death after stimulation with LPS, we used several inhibitors of signaling pathways involved in the induction of apoptosis. Herbizymycin A, a tyrosine kinase inhibitor (24), significantly suppressed LDH release (Fig. 3A). We observed similar inhibitory effects with concentrations up to 20 μM (data not shown). In contrast, SB203580, a p38 MAPK inhibitor (25–27), PD98059, an inhibitor of MEK1 (28), and LY294002, an inhibitor of PI3K (29), failed to suppress LDH release induced by LPS (Fig. 3A). No inhibiting effects were observed for up to 50 μM PD98059 or LY294002 when cells were stimulated with LPS (data not shown). These results suggest that tyrosine kinase activation is involved in LPS-induced cell death in TBBEC. To confirm that herbizymycin A inhibits apoptosis in LPS-treated TBBEC, we analyzed annexin V binding in TBBEC treated with LPS and herbizymycin A. Herbizymycin A inhibited the apoptosis as measured by annexin V binding of LPS-treated TBBEC at 2 h while PD98059 did not inhibit apoptosis in LPS-treated TBBEC (Fig. 3B). Similar results were obtained at 1 and 4 h of LPS stimulation (data not shown).

Next, we performed experiments with several caspase inhibitors. As shown in Fig. 3, Ac-YVAD-CHO (caspase 1 inhibitor), Ac-DEVD-CHO (caspase 3 inhibitor), and Z-Asp-CH2-DCB (pan-caspase inhibitor) suppressed the LDH release induced by LPS. Inhibitory effects were dose dependent for up to 500 μM Ac-YVAD-CMK or Ac-DEVD-CHO and 250 μM Z-Asp-CH2-DCB. These results indicate that caspase activation is involved in the cell death of TBBEC and suggest that the cell death detected by LDH release is apoptosis.

Kinetics of caspase activation and subsequent induction of apoptosis

Next, we tried to detect caspase activity induced by LPS in TBBEC. We detected a significant increase in caspase 3-like activity at 8 h after the addition of LPS and observed an increase in activity up to 16 h (Fig. 4). In addition, caspase 6- or 8-like activity increased at 10 or 12 h, respectively, and continued to increase up to 16 h, but the activity was less than caspase 3-like activity (Fig. 4). No increase in caspase 3-, 6- or 8-like activity was detected without LPS stimulation (Fig. 4). In contrast, caspase 1 or caspase 5-like activity was not detected after stimulation with LPS (Fig. 4).

Signal pathway to induce apoptosis in TBBEC

To clarify what molecular signaling components are involved in LPS-induced activation of caspases, we used several inhibitors. Caspase 3-like activity induced by LPS was significantly suppressed by herbizymycin A or LY294002, but PD98059 had no effect (Fig. 5A). We detected significant inhibition of caspase 6-like activity after addition of herbizymycin A and a strong trend of inhibition after LY294002 (Fig. 5B), but caspase 8-like activity was not inhibited by any of the inhibitors used in Fig. 5 (data not shown). These results suggest that LPS activates caspases through tyrosine kinases and PI3K, which ultimately leads to LDH release.

It has been reported that JNK plays an important role in induction of apoptosis (12, 13). Moreover, our data showed involvement of tyrosine kinase, which is upstream of MAPK, but neither p38 nor ERK1/2 seem to be involved in LPS-induced apoptosis in TBBEC (Figs. 3 and 5). First, we performed experiments with the
JNK inhibitor SP600125. As shown in Fig. 6, SP600125 suppressed cytotoxicity induced by LPS in a dose-dependent manner. Next, we analyzed the effect of SP600125 on LPS-induced caspase activation. Forty micromolar SP600125 inhibited caspase 3- and 6-like activity significantly but not completely (Fig. 7). As shown in Fig. 5, LY294002 suppressed activation of caspases 3 and 6, suggesting that both PI3K and JNK signaling pathways are involved in the induction of apoptosis in TBBEC. Actually, the combination of SP600125 with LY294002 inhibited LPS-induced caspase 3 and 6 activities completely (Fig. 7). These results suggest that LPS induces apoptosis by engaging PI3K and JNK signaling pathways through tyrosine kinase in TBBEC.

Kinetics of mitogen-activated proteins and caspase 3 activation in HMEC

In contrast to TBBEC, LPS does not induce apoptosis in HMEC (7). However, in the presence of the protein synthesis inhibitor CHX, LPS induces apoptosis in HMEC or macrophages (7). Moreover, CHX itself does not induce apoptosis at the concentration used to inhibit de novo protein synthesis (30), suggesting that the LPS signaling pathway is involved in induction of apoptosis. To investigate why HMEC are protected from LPS-induced apoptosis, we analyzed activation of MAPK in response to LPS in the presence or absence of CHX in HMEC. As shown in Fig. 8, phosphorylation of JNK was observed 2 h after the addition of LPS and CHX, and this activation was sustained for 6 h in the case of combined LPS and CHX treatment. Moreover, activation of caspase 3, a typical feature of apoptosis, occurred at 4 h and was sustained for at least 6 h (Fig. 8). These results show that

FIGURE 3. Effects of various inhibitors on LPS-induced cytotoxicity in TBBEC. Cells were pretreated with 0.5% DMSO, 250 μM Ac-YVAD-CHO, Ac-DEVD-CHO, Z-Asp-CH2-DCB, 13.3 μM SB203580, 5 μM herbimycin A, 25 μM PD98059, or 5 μM LY294002 for 1 h and then treated with or without 100 ng/ml LPS for 22 h. Cytotoxicity was assayed by a LDH release assay (A). Results are means ± SE for at least three independent experiments. *, p < 0.02; **, p < 0.01; and ***, p < 0.001. Cells were pretreated with 0.5% DMSO, 5 μM herbimycin A, or 25 μM PD98059 for 1 h and then treated with or without 100 ng/ml LPS for 2 h. Apoptosis was detected by annexin V-fluorescein and propidium iodide staining. Representative photographs of three independent experiments are shown (B).

FIGURE 4. Time course of induction of caspase-like activities in TBBEC treated with LPS. Cells were treated with (A, ●, and ■) or without (△, ○, and □) 100 ng/ml LPS for various time periods as labeled on the abscissa. After incubation, cells were lysed with ICE extraction buffer and caspase-like activities were measured in ICE standard buffer as described in Materials and Methods. The results are means ± SE from four independent experiments. *, p < 0.05.
phosphorylation of JNK precedes cleavage of caspase 3 in HMEC treated with LPS and CHX. On the other hand, activation of p38, ERK1/2, or AKT showed no correlation with the activation of caspase 3 (Fig. 8). These results suggest that the JNK pathway might be involved in the induction of apoptosis in HMEC. To further investigate whether differences in the JNK pathway activation can fully account for these differences in susceptibility to apoptosis, additional experiments were conducted.

FIGURE 5. Effects of various inhibitors on LPS-induced activation of caspase 3 (A) and caspase 6 (B). Cells were treated with 0.5% DMSO, 5 μM LY294002, 25 μM PD98059, or 5 μM herbimycin A for 1 h followed by incubation for 14 h without or with LPS. The results shown are means ± SE from four independent experiments. *, p < 0.05; **, p < 0.02; and ***, p < 0.01.

FIGURE 6. Effect of SP600125 on LPS-induced cytotoxicity in TBBEC. Cells were preincubated with various concentration of SP600125 for 1 h and then treated with 100 ng/ml LPS for 22 h. LDH was measured in the supernatants. The results are means ± SE from four independent experiments. *, p < 0.02.

FIGURE 7. Effects of the combination of LY294002 (LY) and SP600125 (SP) on LPS-induced activation of caspase 3 (A) and caspase 6 (B) in TBBEC. Cells were pretreated with 40 μM SP600125 plus 5 μM LY294002 for 1 h and cultured with 100 ng/ml LPS for 14 h. Then caspase 3 and caspase 6 activities were measured as described in Materials and Methods. The results are means ± SE from three independent experiments. *, p < 0.02; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001.

FIGURE 8. Time course of LPS-induced activation of caspase 3 and MAPK in HMEC. HMEC were treated with or without 100 ng/ml LPS and with or without 40 μg/ml CHX for the indicated times. Western blotting experiments were performed as described in Materials and Methods. One representative experiment of three independent experiments is shown.
between HMEC and TBBEC, we examined phosphorylation of JNK in response to LPS in primary human coronary aortic EC (HCAEC). Consistent with our findings in HMEC, we observed that LPS does only induce minute phosphorylation of JNK in HCAEC. However, treatment of HCAEC with LPS and CHX leads to an increase of JNK phosphorylation (data not shown).

The role of JNK in induction of apoptosis in HMEC treated with LPS and CHX in HMEC

To verify the involvement of JNK in LPS/CHX-induced apoptosis in HMEC, we used an inhibitor of JNK. As shown in Fig. 9, 25 μM SP600125 suppressed phosphorylation of JNK completely, but had no effect on JNK protein expression. Cleaved caspase 3 induced by addition of LPS and CHX was also suppressed by the addition of SP600125 compared with DMSO controls (Fig. 9), suggesting that the JNK pathway is involved in the induction of apoptosis in HMEC by LPS and CHX. In addition, it has been reported that FLIP is induced by LPS itself but CHX suppresses this expression. Since FLIP functions as an inhibitor of caspase 8 activation, we determined whether FLIP expression is altered in LPS- and CHX-treated cells (8). We did not observe a correlation between JNK activation and FLIP expression (Fig. 9). These data suggest that FLIP is not involved in induction of apoptosis induced by LPS and CHX.

Discussion

In this study, we characterized LPS-induced apoptosis in bovine brain EC (TBBEC). LPS-induced signaling pathways leading to apoptosis in TBBEC included tyrosine kinase, JNK, and PI3K activation, but not ERK1/2 or p38 activation. In TBBEC treated with LPS, caspase 3 was activated first, followed by caspase 6 and caspase 8. We did not observe caspase 1- or caspase 5-like protease activity (Fig. 4). These data are in contrast to our experiments using the caspase 1 inhibitor Ac-YVAD-CMK. Ac-YVAD-CMK efficiently suppressed LPS-induced cytotoxicity in TBBEC. Ac-YVAD-CMK inhibits both caspase 1-like and caspase 3-like activity, which explains the discrepancy between our data derived from inhibitor studies and direct measurements of caspase 1 activity (31). These results show that activation of caspase 3 is not dependent on caspase 1 or caspase 8 in LPS-induced TBBEC apoptosis, despite the fact that caspase 1 or 8 is upstream of caspase 3 (32, 33). As shown in Figs. 5 and 6, herbimycin A, SP600125, or LY294002 suppressed LPS induction of caspase 3 or 6, but did not inhibit caspase 8 (data not shown). These results suggest that activation of caspase 3 and caspase 6 are dependent on signaling through JNK and PI3K. Furthermore, time-course experiments of expression of FLIP, an inhibitor of caspase 8 (9), did not show any difference up to 12 h after the addition of LPS in TBBEC (data not shown), suggesting that caspase 8 is not important for LPS-induced TBBEC apoptosis. These results suggest that any contribution of FLIP to LPS-induced apoptosis is minimal.

Our study showed that the PI3K pathway is involved in the activation of caspase 3 or 6. The PI3K signaling pathway, which also includes AKT, is part of a survival signaling pathway (34, 35). One mechanism is that AKT phosphorylates apoptosis signal-regulating kinase 1 and inhibits proapoptotic function in intestinal epithelial cells (34). We observed that AKT is phosphorylated in response to LPS plus CHX in HMEC, conditions that lead to apoptosis in these EC. This is consistent with previous observations that LPS induces both pro- and antiapoptotic pathways in EC (36–39). However, it is still unknown how PI3K and JNK signaling pathways cooperate in the induction of caspase activity in LPS-treated EC.

Furthermore, we demonstrate here that JNK phosphorylation is a common signaling pathway inducing apoptosis in TBBEC and HMEC treated with LPS. Although, LPS directly induced apoptosis in TBBEC, in HMEC LPS alone was unable to induce JNK phosphorylation, but the presence of inhibitors of de novo protein synthesis induces JNK phosphorylation followed by caspase 3 cleavage and apoptosis. Inhibition of JNK activation efficiently blocked caspase 3 cleavage and apoptosis in TBBEC and HMEC. LPS activates proapoptotic and antiapoptotic pathways (7). In HMEC, LPS induced apoptosis in the presence of either CHX or actinomycin D (7). This indicates that de novo mRNA or protein synthesis induced by LPS is necessary for protection from apoptosis, suggesting that LPS itself induces antiapoptotic effects. We have previously demonstrated that the addition of CHX after >30 min of LPS stimulation blocked LPS in macrophages (30), supporting the notion that early de novo protein synthesis induced by LPS has antiapoptotic effects. Previous studies report that FLIP inhibits apoptosis in HMEC and, accordingly, CHX decreases FLIP expression and thereby promotes apoptosis in HMEC (9). However, we did not observe any differences in FLIP protein expression in LPS-treated HMEC, CHX-treated HMEC or LPS plus CHX-treated HMEC (Fig. 9). These results suggest that other molecule(s) are also important for survival of HMEC-treated with LPS.

Recently, phosphorylation of JNK has been reported to be involved in apoptosis induction (40). However, the role of JNK during apoptosis is controversial, with some reports demonstrating that JNK is proapoptotic (40), while others demonstrated protection by JNK (41). Apoptosis signal-regulating kinase 1 was reported as an associated molecule involved in sustained JNK activation (42). We show here sustained phosphorylation of JNK followed by sustained caspase 3 cleavage and HMEC apoptosis (Fig. 8). LPS induces transient activation of JNK (43, 44) followed by NF-κB activation, which regulates cellular survival. In HMEC-treated with LPS plus CHX, we detected sustained degradation of IκBα and NF-κB activation (data not shown) during progression of apoptosis. Sustained NF-κB activation can lead to inflammation-induced tissue damage or malignancy (45), which might suggest that perturbation of NF-κB regulation affects cell survival.

During sepsis, endotoxin released from bacteria affects EC function and leads to induction of cytokines, adhesion molecules, and NO, which enhances sepsis by promoting further inflammation, coagulation, and a decrease of blood pressure (9). In addition, endotoxin induces apoptosis of vessel EC. In this study, we elucidate further the mechanism and molecular signaling by which LPS induces cytotoxicity in vascular EC.
Acknowledgments

We thank Wenxuan Zhang for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

References


