Shiga-like Toxin Inhibition of FLICE-like Inhibitory Protein Expression Sensitizes Endothelial Cells to Bacterial Lipopolysaccharide-induced Apoptosis*

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Shiga-like toxin (SLT) has been implicated in the pathogenesis of hemolytic uremic syndrome and its attendant endothelial cell (EC) injury. Key serotypes of Escherichia coli produce SLT-1 in addition to another highly pro-inflammatory molecule, lipopolysaccharide (LPS). It has previously been established that SLT-1 induces EC apoptosis and that LPS enhances this effect. LPS alone has no affect on human EC viability, and the mechanism for this enhancement remains unknown. In the present report, we demonstrate that SLT-1 sensitizes EC to LPS-induced apoptosis. Pretreatment with SLT-1 sensitized EC to LPS-induced apoptosis, whereas pretreatment with LPS did not influence SLT-1-induced apoptosis. SLT-1 exposure resulted in decreased expression of FLICE-like inhibitory protein (FLIP), an anti-apoptotic protein that has previously been shown to block LPS-induced apoptosis. This SLT-1-mediated decrease in FLIP expression preceded the onset of apoptosis elicited by SLT-1 alone or in combination with LPS. SLT-1-mediated decrements in FLIP expression correlated in a dose- and time-dependent manner with sensitization to LPS-induced apoptosis. Finally, transient or stable overexpression of FLIP protected against LPS enhancement of SLT-1-induced apoptosis, and this protection corresponded with sustained expression of FLIP. Together, these data suggest that SLT-1 sensitizes EC to LPS-induced apoptosis by inhibiting FLIP expression.

EXPERIMENTAL PROCEDURES

Materials—Shiga-like toxin 1 derived from E. coli 0157 was obtained from List Biological Laboratories, Inc. (Campbell, CA). LPS from E. coli 0567–40574, 2002

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serotype 0111:B4, polymyxin B, and dimethyl sulfoxide (Me$_2$SO) were purchased from Sigma. The caspase inhibitor peptide z-VAD-fmk (z-VAD) and the proteasome inhibitors $\beta$-lactone and lactacystin were purchased from Calbiochem-Novabiochem. FLIP$_L$ adenovirus was obtained from Dr. Andrea Gambotto through the University of Pittsburgh Vector Core Reagent Program, Human Gene Therapy Center (Pittsburgh, PA).

Cell Culture—The human dermal microvascular EC line (developed and generously provided by F. J. Candal and Dr. E. Ades, Centers for Disease Control, and Dr. T. Lawley, Emory University, Atlanta, GA) (37) was cultured in RPMI medium (BioWhittaker, Inc., Walkersville, MD) enriched with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), endothelial cell growth factor prepared from bovine hypothalamus (2 ng/ml), sodium pyruvate (1 mM), and nonessential amino acids in the presence of penicillin (100 units/ml) and streptomycin (100 $\mu$g/ml) (all purchased from BioWhittaker).

Immunoblotting—EC monolayers were washed once with phosphate-buffered saline, lysed with ice-cold modified 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 mM vanadate, 50 mM NaF), scraped, transferred to microcentrifuge tubes, and centrifuged (16,000 x $g$, 15 min, 4°C). Total protein was determined using the BCA protein assay (Pierce). The supernatants were combined with 5X sample buffer (Genomic Solutions Inc., Chelmsford, MA), boiled for 3 min, and 20 $\mu$g of protein/lane were resolved by SDS-PAGE on a 4-20% Tris-glycine gradient gel (Novex Inc., San Diego, CA). Protein was subsequently transferred for 1 h at 100 V to polyvinylidene fluoride membrane (Millipore Corp, Bedford, MA). Blots were blocked with 5% dry milk and then incubated with anti-Bcl-2 (0.5 $\mu$g/ml), anti-Bcl-xL (0.25 $\mu$g/ml), anti-Bax (1.0 $\mu$g/ml) (all purchased from Transduction Laboratories Inc., Lexington, KY), or anti-c-FLIP (NPS; 1:20 dilution; generous gift of Dr. Peter H. Krammer of the German Cancer Research Center, Heidelberg, Germany) (38) antibodies for 1 h at room temperature. The blots were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G (IgG) (0.13 $\mu$g/ml; Transduction Labs), developed with enhanced chemiluminescence (Amersham Biosciences), and exposed to Kodak X-Omat Blue film (PerkinElmer Life Sciences). To ensure equal protein loading, select blots were incubated with stripping buffer (Pierce) at 40°C for 20 min, washed, blocked, and re-probed with anti-$\beta$-tubulin murine monoclonal antibody (0.5 $\mu$g/ml; Roche Molecular Biochemicals) followed by horseradish peroxidase-conjugated anti-mouse IgG (0.13 $\mu$g/ml) (Transduction Labs). In select experiments, blots were scanned with a Microtek ScanMaker (Microtek Lab, Inc., Redondo Beach, CA) and analyzed using NIH Image software version 1.62 (National Institutes of Health, Bethesda, MD).

Caspase Assay—For the detection of apoptosis, caspase activity was measured as previously described (39). Briefly, EC were seeded into 96-well plates at a density of 60,000 cells/well, cultured for 24 h, and treated. Caspase activity was measured with a fluorometric caspase assay utilizing the caspase-3 substrate, DEVD, conjugated to rhodamine 110 (Molecular Probes, Eugene, OR) at 485 nm excitation and 530 nm emission, and caspase activity was measured as previously described (39). Briefly, EC were seeded into 96-well plates at a density of 60,000 cells/well, cultured for 24 h, and treated. Caspase activity was measured with a fluorometric caspase assay utilizing the caspase-3 substrate, DEVD, conjugated to rhodamine 110 according to the manufacturer’s instructions (Roche Molecular Biochemicals). The plates were analyzed on a Cytofluor Series 4000 fluorescence plate reader (Perseptive Biosystems Inc., Framingham, MA) at 485 nm excitation and 530 nm emission, and caspase activity was expressed relative to simultaneous medium control.

Cloning and Stable Expression of cDNA Constructs—cDNA encoding the long form of human FLIP, FLIP$_L$, (a generous gift of Dr. Jurg Tschopp, Institute of Biochemistry of the University of Lausanne, Switzerland), was cloned into the bicistronic retroviral expression plasmid, pBMN-IRES-enhanced green fluorescent protein (kindly provided by Dr. Gary Nolan, Stanford University, Stanford, CA) (40). High titer retrovirus was prepared from the Phoenix amphotropic packaging cell line (ATCC, Manassas, VA) transfected with 24 $\mu$g of the expression plasmid by calcium phosphate precipitation. Recombinant retroviral supernatants were collected 48 h after transfection and filtered through a 0.45 $\mu$m filter (Millipore Corp, Bedford, MA). For infection, $4 \times 10^5$ EC were seeded per well of a 6-well plate for 24 h to achieve 80% confluence. The growth medium was replaced with 2.5 ml of retroviral supernatant supplemented with 32 $\mu$g/ml Polybrene and 100 nM HEPES, and the plate was centrifuged for 2 h (1430 x $g$, 32°C). The cells were then incubated for 10 h (5% CO$_2$, 37°C) at which time the retroviral supernatant was replaced with normal growth medium. Cells were analyzed and sorted on the basis of enhanced green fluorescent protein expression using a FACScan Vantage SE cell sorter (BD Biosciences).

Statistical Methods—A t test or analysis of variance was used to compare the mean responses between a single experimental group and its control or among multiple experimental groups, respectively. For experiments analyzed by analysis of variance, the Tukey post hoc comparison test was used to determine between which groups significant differences existed. All statistical analyses were performed using GraphPad Prism version 3.00 for Macintosh (GraphPad Software, Inc., San Diego, CA). A $p$ value of <0.05 was considered significant.

RESULTS

LPS Synergistically Enhances SLT-1-induced Apoptosis—To assay for the ability of LPS and/or SLT-1 to induce apoptosis in EC, caspase activity was measured. Caspases are highly specific effector proteases, the activation of which is a hallmark of apoptosis (41, 42). Consistent with previous reports (7, 20), SLT-1 directly induced EC apoptosis, whereas LPS alone had no effect on EC viability (Fig. 1). EC exposed to both LPS and SLT-1 ($LPS+SLT-1$) demonstrated enhanced caspase activity relative to EC exposed to SLT-1 alone. Pretreatment of EC with the cell-permeable peptide z-VAD, a caspase inhibitor with broad selectivity for several members of the caspase family (43, 44), completely protected against SLT-1- and LPS+SLT-1-induced apoptosis (Fig. 1A).

LPS is composed of both a polysaccharide region and a lipid A moiety, the latter of which is the bioactive portion of the molecule (45). The lipid A moiety of the molecule activates the LPS receptor, Tlr-4, and is responsible for its pro-inflammatory properties (45, 46). Furthermore, it has been reported that in the absence of new gene expression, LPS directly induces EC
apoptosis and that its ability to elicit apoptosis is dependent upon the lipid A moiety (39). To determine whether the enhanced EC apoptosis elicited by LPS/H1100 SLT-1 requires LPS bioactivity, caspase activity was assayed in EC exposed to medium, LPS, SLT-1, or LPS/H1100 SLT-1 in the presence or absence of polymyxin B (Fig. 1B). Polymyxin B, derived from the bacterium Bacillus polymyxa, has been well described to bind to and neutralize the lipid A moiety of LPS (47, 48). Neutralization of LPS with polymyxin B completely inhibited the LPS-induced enhancement of SLT-1-mediated apoptosis (Fig. 1B). In contrast, polymyxin B had no effect on the ability of SLT-1 to directly induce apoptosis.

SLT-1 Pretreatment Sensitizes EC to LPS-induced Apoptosis—After establishing that co-incubation of LPS with SLT-1 dramatically increases EC apoptosis relative to exposure to SLT-1, we next performed an assay to determine whether pretreatment with either SLT-1 or LPS alone could sensitize EC to enhanced killing when subsequently treated with either LPS or SLT-1, respectively (Fig. 2). Pretreatment with LPS had no significant affect on SLT-1-induced killing. Interestingly, pretreatment with LPS inhibited the subsequent LPS enhance-

**Fig. 2.** Effect of SLT-1 pretreatment on sensitization of EC to LPS-induced apoptosis. EC were pretreated for 4 h with medium, LPS (100 ng/ml), or SLT-1 (10 ng/ml), washed twice with medium, subsequently exposed to medium, LPS (100 ng/ml), SLT-1 (10 ng/ml), or LPS+SLT-1 for 8 h, and caspase activity was assayed. Vertical bars represent the mean (±S.E.) caspase activity relative to simultaneous media controls.

**Fig. 3.** Effect of SLT-1 on EC anti-apoptotic protein expression. EC were exposed to medium, LPS (100 ng/ml), SLT-1 (1000 ng/ml), or LPS+SLT-1 for 8 h and lysed. EC lysates were immunoblotted with antibodies raised against the anti-apoptotic proteins, FLIP, Bcl-2, Bcl-x, or the pro-apoptotic protein, Bax. To demonstrate equal protein loading, blots were reprobed with an antibody against β-tubulin (A). In other experiments, EC were pretreated for 1 h with Me2SO, z-VAD (100 μM), or a combination of lactacystin (10 μM) and its aqueous derivative β-lactone (10 μM) (LAC) and subsequently exposed to medium or SLT-1 (1000 ng/ml) for 8 h and lysed (B). Lysates were resolved by SDS-PAGE, transferred to polyvinylidene fluoride membranes, and immunoblotted with anti-FLIP antibody. Molecular mass (in kDa) is indicated.

apoptosis and that its ability to elicit apoptosis is dependent upon the lipid A moiety (39). To determine whether the enhanced EC apoptosis elicited by LPS+SLT-1 requires LPS bioactivity, caspase activity was assayed in EC exposed to medium, LPS, SLT-1, or LPS+SLT-1 in the presence or absence of polymyxin B (Fig. 1B). Polymyxin B, derived from the bacterium Bacillus polymyxa, has been well described to bind to and neutralize the lipid A moiety of LPS (47, 48). Neutralization of LPS with polymyxin B completely inhibited the LPS-induced enhancement of SLT-1-mediated apoptosis (Fig. 1B). In contrast, polymyxin B had no effect on the ability of SLT-1 to directly induce apoptosis.

**Fig. 4.** Dose-dependent effect of SLT-1 on sensitization of EC to LPS-induced apoptosis and decreased FLIP expression. EC were incubated with medium (open circles) or LPS (100 ng/ml) (closed circles) in the presence of increasing concentrations of SLT-1 for 8 h, and caspase activity was assayed (A). Mean (±S.E.) caspase activity is reported relative to simultaneous media controls. In other experiments, EC were incubated with increasing concentrations of SLT-1 for 8 h and lysed (B). EC lysates were resolved by SDS-PAGE, transferred to polyvinylidene fluoride membranes, and immunoblotted with anti-FLIP antibody. Three separate immunoblots were scanned and analyzed, and the results are expressed in arbitrary densitometric units relative to medium control.

**Fig. 4.** Dose-dependent effect of SLT-1 on sensitization of EC to LPS-induced apoptosis and decreased FLIP expression. EC were incubated with medium (open circles) or LPS (100 ng/ml) (closed circles) in the presence of increasing concentrations of SLT-1 for 8 h, and caspase activity was assayed (A). Mean (±S.E.) caspase activity is reported relative to simultaneous media controls. In other experiments, EC were incubated with increasing concentrations of SLT-1 for 8 h and lysed (B). EC lysates were resolved by SDS-PAGE, transferred to polyvinylidene fluoride membranes, and immunoblotted with anti-FLIP antibody. Three separate immunoblots were scanned and analyzed, and the results are expressed in arbitrary densitometric units relative to medium control.
SLT-1 Induces Decreased Expression of FLIP in EC—It has been well described that inhibition of de novo protein synthesis with CHX sensitizes EC to LPS-induced apoptosis (7, 20, 28). We have previously established that in the presence of CHX, expression of the anti-apoptotic protein FLIP is rapidly decreased (20). This decrease in FLIP expression is due to inhibition of de novo synthesis of FLIP coupled with the rapid degradation of pre-existing levels of FLIP via the proteasome. Furthermore, we have shown that decreased expression of FLIP sensitizes EC to direct LPS killing (20). Interestingly, SLT-1 is a well described inhibitor of protein synthesis (9, 36, 53). We, therefore, decided to investigate whether SLT-1 could alter the expression of FLIP. EC treated with SLT-1 (1 μg/ml) demonstrated a profound reduction in FLIP expression relative to EC exposed to medium alone (Fig. 5A). The expression of other known EC anti-apoptotic proteins, including Bcl-2 (54) and Bcl-xL (55), were unaffected by SLT-1 treatment as was expression of the pro-apoptotic Bcl-2 family member, Bax. LPS had no effect on the expression of any proteins screened. EC co-incubated with both LPS and SLT-1 demonstrated a decrement in FLIP expression that was comparable with that observed with EC exposed to SLT-1 alone (Fig. 3A). Caspases are highly specific proteases that cleave numerous cellular substrates during apoptosis (19, 56). To determine whether the decreased level of FLIP could be attributed to caspase-mediated degradation by SLT-1-activated caspases, EC were exposed to SLT-1 in the presence or absence of z-VAD (Fig. 3B). Using the same concentration of z-VAD that completely protected against SLT-1 and LPS+SLT-1-induced apoptosis, z-VAD failed to block the SLT-1-induced decrement in FLIP expression. Therefore, the decrease in FLIP expression was not attributed to SLT-1 activation of caspases.

There are two predominant mechanisms for intracellular degradation of proteins, one involving the lysosomal apparatus and the other involving the proteasome (57). The lysosomal pathway is primarily involved in the proteolytic degradation of membrane-bound proteins and extracellular proteins, whereas the proteasome pathway is primarily responsible for degradation of cytosolic proteins. To determine whether SLT-1-induced decreases in the level of FLIP could be attributed to rapid turnover via the proteasome, EC were pretreated with a highly specific proteasome inhibitor, lactacystin, and its derivative β-lactone (58, 59) and subsequently exposed to medium, LPS, SLT-1, or LPS+SLT-1 (Fig. 3B). Proteasome inhibition significantly protected against decreased levels of FLIP in the presence of SLT-1. These data suggest that SLT-1 inhibits de novo synthesis of FLIP and that existing levels of FLIP are rapidly depleted through a degradation process involving the proteasome. This latter finding is compatible with two previous studies demonstrating a role for the proteasome in mediating FLIP degradation (60, 61). In those studies, p53- and peroxisome proliferator-activated receptor γ modulator-mediated down-regulation of FLIP was blocked by specific inhibition of the proteasome (60, 61), consistent with the findings here that proteasome inhibition with lactacystin protects against SLT-1-mediated decrements in FLIP expression (Fig. 3B). Furthermore, Kim et al. (61) report that ubiquitination of FLIP, a requisite step in proteasome-mediated degradation, coincided with the decrease in FLIP expression that was elicited by a peroxisome proliferator-activated receptor γ modulator. Although we were unable to detect ubiquitinated forms of FLIP after SLT-1 treatment, the finding that lactacystin inhibits the SLT-mediated decrement in FLIP expression suggests a role for the proteasome in mediating this event.

SLT-1 Inhibition of FLIP Expression Correlates with Sensitization to LPS-induced Apoptosis in a Dose- and Time-dependent Manner—EC treated with SLT-1 or LPS+SLT-1 demonstrated a dose-dependent increase in caspase activity that was maximal at 10 ng/ml SLT-1 (Fig. 4A). The lowest dose of SLT-1 assayed, 1 pg/ml, failed to induce apoptosis relative to medium alone; however, in combination with LPS, this dose of SLT-1 induced EC apoptosis. Analysis of lysates derived from EC exposed to increasing concentrations of SLT-1 revealed a dose-dependent decrease in FLIP expression (Fig. 4B). Densitometric analysis revealed a decrease in FLIP expression in EC exposed to SLT-1 doses as low as 1 pg/ml (Fig. 4B), a concentration that sensitized EC to LPS+SLT-1 killing. EC treated for increasing periods of time with a fixed concentration of SLT-1 (10 ng/ml) in the presence or absence of LPS demonstrated a time-dependent increase in caspase activity at ≥4 h (Fig. 5A). Decreased expression of FLIP was evident in EC exposed to SLT-1 for 2 h (Fig. 5B), a time point that preceded the onset of apoptosis induced by either SLT-1 alone or LPS+SLT-1. A more dramatic decrease in FLIP expression was observed after a 4-h incubation with SLT-1. This exposure time
preceded the LPS-elicited enhancement of SLT-induced apoptosis that was seen at ≥6-h exposures.

Overexpression of FLIP Protects against LPS+SLT-1-induced Apoptosis—Because SLT-1-mediated decreases in FLIP expression correlated with LPS+SLT-1-induced apoptosis, we hypothesized that increasing pre-existing EC levels of FLIP would protect against SLT-1 sensitization to LPS-induced apoptosis. Adenoviral-mediated transient overexpression of the short isotype of FLIP (FLIPS) (Fig. 6A), which contains just the two death effector domain regions necessary for its anti-apoptotic abilities (62, 63), completely protected against LPS+SLT-1-induced apoptosis (Fig. 6B). Interestingly, expression of FLIPS also protected against SLT-1-elicited apoptosis. This protection corresponded with sustained expression of FLIPS in the presence of SLT-1 (Fig. 6C). Expression of the endogenous long form of FLIP, FLIP_L, was dramatically decreased after SLT-1 exposure as expected. Finally, a dose-dependent relationship was observed between increasing FLIP-containing adenoviral multiplicity of infection and the level of protection conferred (Fig. 6D).

Using a retroviral infection system, the predominant form of FLIP found in EC, FLIP_L (20, 64), was stably overexpressed in human microvascular EC (Fig. 7A). The long form of FLIP contains two death effector domain regions as well as a catalytically inactive caspase-like domain that is homologous to the active site of caspase-8 (62, 63). The enhanced caspase activity elicited by co-incubation with LPS+SLT-1 relative to exposure to SLT-1 alone was inhibited by ~50% in EC stably expressing FLIP_L (Fig. 7B). Protection against LPS+SLT-1-induced caspase activation corresponded with sustained expression of FLIP_L in the presence of SLT-1 (Fig. 7C). In contrast to the FLIPS, FLIP_L failed to offer any protection against SLT-1-evoked apoptosis.

**DISCUSSION**

Shiga toxin is well established to induce EC apoptosis (6, 7, 65, 66). Furthermore, Shiga toxin-induced EC apoptosis is synergistically enhanced by LPS; however, the mechanism by which this occurs remains unknown (7). SLT-1 is structurally, antigenically, and functionally similar to Shiga toxin (1, 67). SLT-1 and Shiga toxin share 98% homology and differ by only 1 amino acid. SLT-1 is neutralized by antiserum to Shiga toxin, and both toxins utilize the same cellular EC receptor for internalization, globotriaosylceramide. Similar to Shiga toxin, SLT-1 has been shown to induce EC apoptosis (9, 10), and in the present report this effect has been demonstrated to be
Synergistically enhanced by LPS (Fig. 1).

To quantify relative changes in apoptosis, we used a caspase activity assay to measure the cleavage of the caspase-3 substrate, DEVD (39). The activation of effector caspases, including caspase-3, initiates a series of highly specific proteolytic cleavage events that leads to the onset of apoptosis (41, 42). The observed increase in caspase activity after SLT-1 exposure (Fig. 1) is consistent with previous reports that SLT-1 induces EC apoptosis as assayed by annexin V staining (9), * significantly decreased relative to identically treated enhanced green fluorescent protein-expressing EC.

We have previously established that under resting conditions EC are resistant to LPS-induced apoptosis (20, 28, 29, 39). However, in the presence of actinomycin D or CHX, inhibitors of mRNA and protein synthesis, respectively, EC are sensitized to LPS-induced apoptosis (20, 28, 29, 39). The fact that LPS induces apoptosis in the absence of new gene expression precludes the possibility that this response was mediated by contaminating LPS in the SLT-1 preparations derived from E. coli. Similar to CHX, Shiga toxin and SLT-1 have been clearly established to inhibit protein synthesis (9, 36, 53, 68). These toxins cleave a specific bond in the 28 S rRNA component of the 60 S ribosomal subunit, resulting in the release of a single adenine base and the inhibition of aminoacyl tRNA binding to protein synthesis (41, 42).

Under physiological conditions, constitutive expression of FLIP confers resistance to LPS-induced apoptosis in human EC. We have established that under resting conditions constitutive expression of FLIP confers resistance to LPS-induced apoptosis in human EC. In the presence of SLT-1, de novo synthesis of FLIP is inhibited. Pre-existing molecules of FLIP are rapidly degraded via the proteasome. The end result of SLT-1 exposure is diminished expression of FLIP and sensitization of EC to LPS-induced apoptosis.

We have established that the bioactive moiety of LPS, lipid A, is responsible for its ability to synergistically enhance SLT-1-induced apoptosis. Neutralization of lipid A with polymyxin B completely inhibited the LPS enhancement of SLT-1-induced apoptosis (Fig. 1B). This is consistent with a previous report that identified lipid A as the portion of the LPS molecule that initiates pro-apoptotic signaling (39). Furthermore, these data are compatible with the finding that deacylation of the fatty acid chains in the lipid A region abolishes LPS enhancement of Shiga toxin-induced EC apoptosis (7).

In the present report, highly purified LPS, which was phenol-extracted and purified by ion exchange chromatography, was used for all experiments. The finding that polymyxin B completely blocked the LPS enhancement of SLT-1-induced apoptosis rules out that this response was influenced by contaminants in the LPS preparation. Furthermore, the observation that polymyxin B had no inhibitory effect on SLT-1-induced apoptosis precludes the possibility that this response was mediated by contaminating LPS in the SLT-1 preparations.
the ribosome (1, 67). Consistent with its role in inhibiting protein synthesis, SLT-1 dose- and time-dependently induced a decrease in FLIP expression (Fig. 4B and 5B). Proteasome inhibition protected against the SLT-1-mediated decrease in FLIP expression (Fig. 3B), suggesting that SLT-1 inhibits de novo protein synthesis and that existing FLIP molecules are rapidly degraded via the proteasome in a manner analogous to CHX. SLT-1 had no effect on the expression of other known anti-apoptotic molecules constitutively expressed in EC (Fig. 3A), including Bcl-2 or Bcl-x, which have long half-lives (20). In one report, up-regulation of the pro-apoptotic protein Bax in epithelial cells has been shown to correlate with SLT-1-induced apoptosis (69). In that study, Bax up-regulation was observed after a 24-h exposure to SLT-1. In this study, SLT-1-induced EC apoptosis was evident within 8 h of exposure to SLT-1, an exposure time that precluded a change in Bax expression (Fig. 3A).

The finding that SLT-1 decreases FLIP expression in combination with the previous report that decreased expression of FLIP sensitizes EC to LPS-induced apoptosis suggested that SLT-1 may sensitize EC to LPS-induced apoptosis by decreasing the expression of FLIP. Consistent with this hypothesis, pretreatment of EC with SLT-1 for 4 h, a time frame compatible with the SLT-1-mediated decrement in FLIP expression (Fig. 5B), resulted in sensitization to LPS-induced apoptosis (Fig. 2). The caspase activity induced by LPS alone after EC pretreatment with SLT-1 was comparable with that observed in EC exposed simultaneously to both LPS and SLT-1. In contrast, pretreatment of EC with LPS failed to significantly enhance SLT-1-induced apoptosis. Pretreatment with LPS did inhibit the ability of subsequent LPS exposure to enhance SLT-induced apoptosis. This finding is compatible with the well described ability of LPS to induce a state of tolerance to its own actions (51, 52). Several studies report that an initial exposure to LPS renders cells hyporesponsive to subsequent LPS treatment by disrupting LPS signaling pathways (49–52). Together, these data suggest that SLT-1 sensitizes EC to LPS-induced apoptosis. The finding that SLT-1 confers responsiveness to an LPS-elicited response is consistent with an in vivo study in which Shiga toxin pretreatment sensitized mice to the lethal effect of LPS (34). In contrast, pretreatment with LPS had no effect on the ability of Shiga toxin to induce lethality.

One previous study examining the synergistic effect of LPS on Shiga toxin-induced cytotoxicity reported that pretreatment of EC with LPS enhanced subsequent SLT-1-induced killing (7). The experiments presented in that study differed from those presented here in several ways. First, the authors used a neutral red assay that measures only cell viability and does not discriminate between necrotic and apoptotic cell death. Second, enhancement of SLT-1-induced apoptosis was observed after a 24-h pretreatment with LPS using a dose that is 10-fold higher than that used in the present studies. The significance of this latter finding is that at this higher concentration, LPS can activate cells in a receptor-independent manner (70). Therefore, the mechanism by which EC respond to LPS under such conditions is difficult to evaluate. Third, those studies were conducted with macrovascular-derived human umbilical vein EC, which reportedly express low levels of the SLT-1 receptor globotriaosylceramide and display low sensitivity to SLT-1 (71). In contrast, we have used EC derived from the microvasculature. Microvascular EC are a principal target of SLT-1 in vivo (1). In contrast to EC derived from large vessels, microvascular EC have higher globotriaosylceramide expression and increased sensitivity to SLT-1 (71, 72). Furthermore, in parallel studies between human umbilical vein EC and microvascular EC, LPS synergistically enhanced SLT-1-induced cytotoxicity in the macrovascular-derived human umbilical vein EC only, not in the microvascular EC (71).

Several lines of evidence suggest that the SLT-1-mediated decrease in FLIP expression sensitizes EC to LPS-induced apoptosis. First, SLT-1 pretreatment for a period of time that results in decreased expression of FLIP sensitizes EC to subsequent LPS-induced apoptosis (Fig. 2). Second, decreased expression of FLIP was evident with 1 pg/ml of SLT-1 (Fig. 4B), a dose that had no effect alone on EC apoptosis, but in combination with LPS induced apoptosis (Fig. 4A). Third, the SLT-1-mediated decrease in FLIP expression was clearly evident after a 4-h exposure, a time point that preceded the synergistic enhancement of SLT-1-induced apoptosis by LPS (Fig. 5).

Fourth, adenoviral-mediated overexpression of FLIPS, which contains the requisite death effector domain regions necessary for its anti-apoptotic ability, inhibited LPS+SLT-1-induced apoptosis (Fig. 6). The inhibition of LPS+SLT-1-induced apoptosis corresponded with sustained expression of FLIP in the presence of SLT-1. The endogenous form of FLIP, FLIPL, was decreased in the adenoviral-transduced EC after SLT-1 treatment. This latter finding demonstrates that adenovirus infection alone does not alter the host cell machinery necessary for normal protein degradation and suggests a specific effect of the overexpressed FLIPS. Finally, stable overexpression of the endogenous form of FLIP constitutively expressed in EC, FLIPs, significantly protected against LPS+SLT-1-induced apoptosis (Fig. 7). This protection correlated with sustained expression of FLIPs in the presence of SLT-1. Together, these data suggest that the synergistic enhancement of SLT-1-induced apoptosis by LPS is mediated by a SLT-1-induced decrement in FLIP expression that sensitizes EC to LPS-induced apoptosis (Fig. 8).

Because SLT-1 elicits a dose- and time-dependent decrease in FLIP that correlates with EC sensitivity to apoptosis induced by SLT-1 alone, the possibility exists that FLIP protects against SLT-1-induced apoptosis. Consistent with this hypothesis, adenoviral-mediated overexpression of FLIPs protected against SLT-1-induced apoptosis (Fig. 6B). Increasing the infectivity (multiplicity of infection) of FLIP vector-containing adenovirus inhibited SLT-1-induced apoptosis in a concentration-dependent manner (Fig. 6D). In contrast, stable overexpression of FLIPs had no cytoprotective effect on SLT-induced apoptosis (Fig. 6B). This latter observation is consistent with a prior report that SLT-induced apoptosis is mediated by Bcl-2 (69). Although adenoviral- or retroviral-mediated overexpression of the FLIP isoforms consistently protected against LPS+SLT-induced apoptosis, the reason for the discrepancy in protection conferred against SLT-1-induced apoptosis remains unknown. Thus, a role for FLIP in mediating EC apoptosis evoked by SLT-1 alone remains unclear.

In the present report, we have provided evidence that the SLT-1-mediated decrease in FLIP expression sensitizes EC to LPS-induced apoptosis. Furthermore, this acquired sensitization to LPS-induced apoptosis provides a mechanism for the synergistic enhancement of SLT-1-induced apoptosis by LPS (Fig. 8). Further studies will be needed to elucidate the signaling pathways by which SLT-1 alone induces apoptosis.

REFERENCES
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