A Constitutive Cytoprotective Pathway Protects Endothelial Cells from Lipopolysaccharide-induced Apoptosis*

Received for publication, January 29, 2001

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Lipopolysaccharide (LPS) has been implicated as the bacterial component responsible for much of the endothelial cell injury/dysfunction associated with Gram-negative bacterial infections. Protein synthesis inhibition is required to sensitize the endothelium to lipopolysaccharide-induced apoptosis, suggesting that a constitutive or inducible cytoprotective protein(s) is required for endothelial survival. We have identified two known endothelial anti-apoptotic proteins, c-FLIP and Mcl-1, the expression of which is decreased markedly in the presence of cycloheximide. Decreased expression of both proteins preceded apoptosis evoked by lipopolysaccharide + cycloheximide. Caspase inhibition protected against apoptosis, but not the decreased expression of c-FLIP and Mcl-1, suggesting that they exert protection upstream of caspase activation. Inhibition of the degradation of these two proteins with the proteasome inhibitor, lactacystin, prevented lipopolysaccharide + cycloheximide-induced apoptosis. Similarly, lactacystin protected against endothelial apoptosis induced by either tumor necrosis factor-α or interleukin-1β in the presence of cycloheximide. That apoptosis could be blocked in the absence of new protein synthesis by inhibition of the proteasome degradative pathway implicates the requisite involvement of a constitutively expressed protein(s) in the endothelial cytoprotective pathway. Finally, reduction of FLIP expression with antisense oligonucleotides sensitized endothelial cells to LPS killing, demonstrating a definitive role for FLIP in the protection of endothelial cells from LPS-induced apoptosis.

Despite advances in anti-microbial therapy and overall medical care, Gram-negative bacterial sepsis remains a common, life-threatening event. The challenge of managing septic patients is compounded by the development of key vascular complications including, systemic vascular collapse, disseminated intravascular coagulation, and vascular leak syndromes (1–5). A common denominator to all these complications is endothelial cell injury/dysfunction associated with Gram-negative bacteria, has been implicated as the causative agent responsible for EC dysfunction (1, 5–7). In the absence of non-endothelial cell-derived host mediator systems, LPS directly evokes numerous EC responses including: 1) up-regulation of adhesion molecules; 2) increased production of cytokines, nitric oxide, and tissue factor; 3) loss of monolayer integrity and barrier function; and 4) apoptosis (8). In addition to a direct role, LPS stimulates the production of inflammatory cytokines, including interleukin (IL)-1β and tumor necrosis factor (TNF)-α, which also elicit an altered pathophysiological endothelial state (9).

LPS-induced EC apoptosis has been observed both in vitro (10–14) and in vivo (15, 16). LPS directly induces apoptosis in bovine and ovine EC (10, 12, 13, 17). Sensitization of human EC to LPS-induced apoptosis requires the inhibition of either mRNA or protein synthesis (18). This latter finding suggests that either a constitutively expressed protein with a relatively short half-life or an inducible protein is requisite for EC survival following LPS exposure. Similarly, two LPS-inducible cytokines, IL-1β and TNF-α, induce human EC apoptosis only when new gene expression is blocked (18).

Apoptosis has been implicated as an important mechanism of in vivo cell death following LPS exposure. Tissues and organs obtained from either patients who have died of sepsis and multi-organ failure (19) or animal models of endotoxiaemia and sepsis reveal enhanced apoptotic cell death (16, 20, 21). The vascular endothelium is one tissue that is sensitive to LPS-induced apoptosis. In a murine model of sepsis, apoptotic EC have been detected in pulmonary capillaries (20). Intravenous administration of endotoxin into rabbits or rats induces EC death and detachment from the artery wall (22, 23). In mice challenged with either LPS or TNF-α, disseminated EC apoptosis has been reported in the lung, thymus, and intestine (15, 16, 24). Finally, injection of a broad spectrum caspase inhibitor following LPS administration decreased EC apoptosis in the lung and improved survival in a murine model of acute lung injury (24). Together, these in vitro and in vivo studies indicate that the vascular endothelium is a key target of LPS-induced apoptosis.

The mechanisms by which LPS activates apoptosis remain unknown. Efforts to elucidate LPS signaling pathways, apoptotic or otherwise, have been hampered by the lack of an identifiable membrane-bound receptor capable of signal trans-
duction. Recently, Toll-like receptor (TLR)-4 has been identified in both cells of monocyte lineage and EC as the receptor responsible for LPS activation of the NF-κB signaling pathway (25–27). Interestingly, MyD88, a TLR-4-binding protein that has a requisite role in the downstream activation of NF-κB in EC, contains a death domain (DD) (28, 29). DD are conserved regions of amino acids, which facilitate protein-protein interaction. In the well characterized TNF pathway, the TNF receptor-binding protein, TRADD, recruits another adapter protein, FADD, through the interaction of their respective death domains. An additional conserved sequence in FADD, the death effector domain (DED), enables the recruitment of caspase 8, an upstream cysteine protease whose activation initiates a cascade of proteolytic events characteristic of apoptosis. We have previously reported that LPS-induced EC apoptosis is FADD-dependent (11). Whether FADD is recruited to MyD88 following LPS stimulation via binding of their respective DD remains unknown.

The anti-apoptotic pathways utilized by human EC to resist LPS-induced apoptosis have yet to be elucidated. Human EC sensitivity to LPS-induced apoptosis is dependent on new protein or mRNA synthesis inhibition, suggesting that either an inducible or constitutively expressed protein is responsible for protection (30). LPS has previously been shown to up-regulate two cytoprotective proteins, the Bcl-2 homologue, A1, and the zinc-finger protein, A20, in EC (30). Overexpression of A1, A20, or Bcl-xL, confers partial protection against LPS and cycloheximide (CHX)-induced apoptosis. There is doubt, however, concerning the physiological relevance of these overexpression studies. Similar to LPS, sensitization of human EC to TNF-α-induced apoptosis requires inhibition of gene expression. Further, overexpression of A1 or Bcl-xL protects EC against TNF-α and CHX induced-apoptosis (31). The selective inhibition of A1 or Bcl-xL, gene expression, however, fails to sensitize human EC to direct TNF-α-induced apoptosis, indicating that these proteins are not critical for protection (32). Another proposed mechanism of protection is through NF-κB-dependent gene expression of certain cytoprotective proteins, including cellular inhibitor of apoptosis proteins (cIAP) (33). Selective blockade of the NF-κB signaling pathway by expression of a mutant IκBα sensitizes EC to apoptosis induced by prolonged exposure to TNF-α, but not LPS (14). This finding suggests that cell survival following LPS exposure is NF-κB-independent. In the present report, we have attempted to elucidate the mechanism of human EC resistance to LPS-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—The caspase inhibitor peptide, z-VAD fluoromethylketone (z-VAD), CHX, and the proteasome inhibitors, b-lactone and lactacystin, were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Staurosporine was obtained from Kamiya Biomedical Co. (Seattle, WA). LPS from *Escherichia coli* serotype 055:B5 and dimethyl sulfoxide (Me2SO) were purchased from Sigma. Recombinant human TNFα and IL-1β were purchased from R&D Systems, Inc.

**EC Culture**—Human umbilical vein EC were obtained from collagenase-digested umbilical veins and cultured in RPMI medium (Biowhittaker) supplemented with 10% fetal bovine serum and incubated with 5 μg/ml anti-VCAM-1 monoclonal antibody (clone 4B9) for 1 h at 37°C. Monolayers were washed as above, fixed for 10 min with 0.05% glutaraldehyde in PBS, washed again, and incubated with HRP-conjugated anti-mouse IgG (1 mg/ml) (Transduction Laboratories, Inc., Lexington, KY). Antibody was added to monolayers for 1 h at room temperature, rinsed, and then developed with 0.05% 5-bromo-4-chloro-3-indolyl phosphate and 0.02% nitroblue tetrazolium (Sigma). Monolayers were scored as positive when blue staining was present. Monolayers were seeded into 96-well plates at a density of 20,000 cells/well for 24 h. Following treatment, cells were washed twice with RPMI media supplemented with 2.5% bovine serum and incubated with 5 μg/ml anti-VCAM-1 monoclonal antibody (clone 4B9) for 1 h at 37°C. Monolayers were washed as above, fixed for 10 min with 0.05% glutaraldehyde in PBS, washed again, and incubated with HRP-conjugated anti-mouse IgG (1 mg/ml) (Transduction Laboratories) for 1 h at 37°C. EC were then washed five times with PBS, developing solution (1 mg/ml o-phenylenediamine in 0.1 M sodium citrate (pH 4.5) with 0.003% hydrogen peroxide) added for 40 min at room temperature, and plates analyzed at 490 nm on a microplate reader (Bio-Tec Instruments, Inc., Winooski, VT). Background readings determined as EC exposed to medium alone were subtracted from each experimental treatment.

**Vascular Cell Adhesion Molecule-1 (VCAM-1) Enzyme-linked Immunosorbent Assay**—EC were seeded into 96-well plates at a density of 20,000 cells/well and cultured for 48 h. Following treatment, cells were washed twice with RPMI media supplemented with 2.5% bovine serum and lactacystin, were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Staurosporine was obtained from Kamiya Biomedical Co. (Seattle, WA). LPS from *Escherichia coli* serotype 055:B5 and dimethyl sulfoxide (Me2SO) were purchased from Sigma. Recombinant human TNFα and IL-1β were purchased from R&D Systems, Inc.

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**Adenoviral Transduction**—The IκBα mutant (IκBαM) and β-galactosidase recombinant adenoviruses were gifts of Dr. C. B. Wilson (University of Washington, Seattle, WA). The IκBαM (S22A/S26A) cDNA was generously provided by Drs. J. Di Donato and M. Karin (University of California, San Diego, CA) (40). Construction and purification of IκBαM and control (β-gal) adenoviruses were performed as described (14). For adenoviral transduction, EC were seeded into 96-well plates at a density of 8,000 cells/well for 24 h, washed once with medium, and incubated for 72 h at a multiplicity of infection of 1000 with control or IκBαM adenoovirus in complete EC medium. EC were washed once with complete medium and subjected to experimental treatment.
Oligonucleotide Design and Synthesis—2′-O-Methoxyethyl/2′-deoxynucleotide chimeric antisense oligonucleotides (32, 41) were used in all experiments. Chimeric oligonucleotides were used to support an RNase H-dependent mechanism of action, which results in a selective loss of target mRNA (42). All oligonucleotides were synthesized and purified as described previously (32, 41). The c-FLIP and Mcl-1 antisense oligonucleotides, respectively, were identified by screening a series of different antisense oligonucleotides designed to hybridize to their respective targets using quantitative reverse transcription-polymerase chain reaction and Northern blot assays (43). ISIS 23296 and ISIS 20408 were found to be the most effective inhibitors of c-FLIP and Mcl-1 expression, respectively. The control antisense oligonucleotide contained eight or four mismatches, as compared with FLIP and Mcl-1 antisense oligonucleotides, respectively. Sequence of the oligonucleotides used are shown in Table I.

**RESULTS AND DISCUSSION**

**Time-dependent Effect of LPS-induced EC Apoptosis in the Absence of New Protein Synthesis—** Exposure to LPS + CHX induced EC death in a time-dependent manner (Fig. 1A). An 8-h stimulus-to-response lag time was observed following LPS + CHX treatment. EC exposed to CHX alone demonstrated no change in viability over this time period, consistent with the ability of CHX to inhibit de novo protein synthesis and cell proliferation without inducing EC death. Increased metabolic activity in EC exposed to medium or LPS alone reflected continued EC proliferation over time. To confirm that the decreased EC viability following administration of LPS + CHX was due to apoptosis, cleavage of PARP and histone release into the cytoplasm were assayed (Fig. 1, B and C). PARP is a known substrate of caspases, a family of highly specific proteases activated during apoptosis (38). Western blot analysis revealed PARP cleavage only in lysates derived from EC exposed to LPS in combination with CHX. Consistent with these data, Hu et al. (30) reported that EC treated with LPS alone or CHX alone demonstrate no evidence of apoptosis, but upon co-administration of both LPS and CHX, cleavage of DNA into repeating oligonucleosomal fragments of 180 base pairs, a hallmark of apoptosis, was observed. Cleavage of PARP was time-dependent and observed at exposures of ≥12 h. Another hallmark of apoptosis is the release of histones into the cytoplasm

### Table I

**Sequence of the c-FLIP, Mcl-1, and control oligonucleotides**

| Target | Sequence (5′→3′) |
|--------|------------------|
| c-FLIP | ACTTGGCTCCTGCTCTTGA |
| Control | TCTAGGCCCTCCTGCTGTAGTA |
| Mcl-1 | TGGGCTTTGTGCTCTTGCCG |
| Control | TGGGACTCTGTTTCTTTGGCCG |

### Figure 1

**Time-dependent effect of LPS + CHX on EC apoptosis.** EC were incubated with medium or LPS (100 ng/ml) in the presence or absence of CHX (40 μg/ml) for increasing exposure times (A–C). EC viability was measured using the Alamar Blue assay of metabolic activity (A). Mean ± S.E. metabolic activity is reported in arbitrary fluorescent units. * significantly decreased compared with all other conditions at the same time point as well as the same treatment at the 4-h time point. In other experiments, EC lysates were immunoblotted with an antibody raised against PARP, a caspase substrate cleaved during apoptosis (B). Molecular mass (in kDa) is indicated. A quantitative assay for apoptosis, which measures histone release into the cytosol, was performed (C). Mean ± S.E. histone release is reported in OD units. * significantly increased compared with all other conditions at the same time point.

(39). Using a highly sensitive ELISA-based assay, the presence of cytosolic mono- and oligonucleosomes were only detected in EC exposed to both LPS and cycloheximide for ≥12 h (Fig. 1C), consistent with the detection of PARP cleavage.

The requisite inhibition of de novo protein synthesis necessary for EC sensitization to LPS-induced apoptosis has been reported for other inflammatory stimuli as well, including TNF-α and IL-1β (14). Further, LPS or TNF-α in the presence of actinomycin D, an inhibitor of mRNA synthesis, induces EC apoptosis suggesting that inhibition of gene expression regardless of whether it occurs at the protein or mRNA level is sufficient for sensitization (18). The ability of LPS to induce EC apoptosis only in the absence of new gene expression implies that either an inducible or constitutively expressed cytoprotective protein is required for EC resistance to LPS-evoked cell death.

**Western Blot Analysis of Anti-apoptotic Proteins following EC Exposure to LPS and CHX—** That inhibition of de novo protein synthesis sensitizes EC to LPS-induced apoptosis suggests that either a rapidly degraded constitutive protein and/or an inducible protein confer resistance under physiological conditions. We, therefore, screened EC exposed to medium, LPS, CHX, or LPS + CHX for changes in the expression of known anti-apoptotic proteins constitutively expressed in human EC, including Akt (44), c-FLIP (45), Mcl-1 (32), Bcl-xL (32), and Bcl-2 (46). The expression of two proteins, FLIP and Mcl-1, was
shown to decrease in the presence of CHX (Fig. 2A). The expression of all other cytoprotective proteins assayed, as well as the pro-apoptotic protein, Bax, remained stable even after a 16-h exposure. Decreases in the protein levels of both FLIP and Mcl-1 were demonstrated within 2 h of CHX exposure, a time frame that precedes the onset of LPS + CHX-induced apoptosis (Fig. 2B).

Interestingly, exposure of EC to both LPS and CHX resulted in a greater decrease in Mcl-1 expression at longer time points (≥4 h) than EC exposed to CHX alone. This latter finding may suggest that, after activation of EC apoptosis by LPS, Mcl-1 becomes a downstream target of caspases. In contrast, diminished levels of FLIP were comparable in EC exposed to CHX alone or in combination with LPS.

FLIP is an anti-apoptotic protein with significant homology to caspase-8 (47). A substitution of two amino acids in the region of FLIP that corresponds to the catalytic active site of caspase-8 renders it incapable of proteolysis. The role of FLIP in the inhibition of FAS death signaling has been well elucidated. Upon Fas ligand binding, the adapter protein FADD is recruited to the Fas receptor via an interaction between the death domains of each protein (48). FADD, in turn, can recruit FLIP and pro-caspase-8 via protein-protein interactions of death effector domains contained within all three proteins. Pro-caspase-8 has pro-caspase-8 activity prevents the transactivation of pro-caspase-8 (34). Alternatively, FADD recruitment of both pro-caspase-8 and FLIP has been suggested to block assembly of a functional death signaling complex (51).

Activation of caspase-9 results in a cascade effect on the activation of effector caspases culminating in apoptosis. Reportedly, Bcl-2 can also block killing downstream of and independent of cytochrome c release (58).

Mcl-1, unlike other Bcl-2 members, contains two PEST sequences, which target it for rapid turnover via proteasome-mediated degradation (54). Correspondingly, inhibition of new protein synthesis resulted in decreased expression of Mcl-1, but no change in the expression levels of other Bcl-2 family members, including Bcl-2, Bcl-xL, and Bax (Fig. 2A). The implication of a cytoprotective role for Mcl-1 is consistent with previous reports that overexpression of other Bcl-2 homologues, including Bcl-xL and A1, confers partial protection against LPS- or TNF-α-induced EC apoptosis (30, 31). An essential protective role, however, for Bcl-xL and A1 at physiological levels is uncertain as selective inhibition of their expression by antisense oligonucleotides fails to sensitize EC to TNF-α-mediated killing (32).

During LPS-induced EC apoptosis, cellular proteins are cleaved after caspase activation (10). To determine whether the decreased levels of FLIP and Mcl-1 could be attributed to caspase-mediated proteolytic degradation, EC were exposed to LPS + CHX in the presence or absence of z-VAD, a highly specific caspase inhibitor with broad selectivity for several members of the caspase family (59, 60), completely protected against LPS + CHX-induced EC apoptosis (Fig. 3, A and B). Caspase inhibition blocked decreased EC viability (Fig. 3A), cleavage of the caspase substrate, PARP (Fig. 3B), and histone release (data not shown) in response to LPS + CHX exposure. These findings are in agreement with previous reports that LPS + CHX-induced EC death is apoptotic in nature and mediated through caspase activation (11).

The TLR family of receptors has been implicated in LPS signaling (25, 52). An adapter protein that plays an integral role in TLR signaling, MyD88, contains a conserved DD (28). We have previously reported that another DD-containing protein, FADD, is required for LPS + CHX-induced apoptosis (11).

A recent report suggests that MyD88 can interact with FADD through reciprocal binding of their DD (53). Analogous to the Fas pathway, the involvement of FADD in the LPS death pathway is compatible with a protective role for FLIP.

Mcl-1 is an anti-apoptotic member of the Bcl-2 family of proteins primarily localized to mitochondrial membranes (54). The Bcl-2 family contains both pro- and anti-apoptotic members capable of heterodimerization. In response to certain apoptotic stimuli, the relative ratios of these pro- and anti-apoptotic proteins can dictate whether a cell will survive or undergo apoptosis (55). Bcl-2 exerts its protection by preventing the release of cytochrome c from the mitochondrion, the latter being a co-factor with Apaf-1 in the activation of procaspase-9 (56, 57). Activation of caspase-9 results in a cascade effect on the activation of effector caspases culminating in apoptosis. Reportedly, Bcl-2 can also block killing downstream of and independent of cytochrome c release (58).

Caspase Inhibition Blocks LPS-induced Apoptosis but Fails to Protect against Decreased FLIP and Mcl-1 Expression—The cell-permeable peptide, z-VAD, a highly specific caspase inhibitor with broad selectivity for several members of the caspase family (59, 60), completely protected against LPS + CHX-induced EC apoptosis (Fig. 3, A and B). Caspase inhibition blocked decreased EC viability (Fig. 3A), cleavage of the caspase substrate, PARP (Fig. 3B), and histone release (data not shown) in response to LPS + CHX exposure. These findings are in agreement with previous reports that LPS + CHX-induced EC death is apoptotic in nature and mediated through caspase activation (11).

The caspase-independent decrease of FLIP and Mcl-1 is consistent with the decrease in their expression preceding both cleavage of PARP, a known caspase substrate, and the onset of apoptosis. Furthermore, CHX exposure alone decreased the expression levels of both FLIP and Mcl-1 following CHX exposure is enhanced with the co-administration of LPS, suggesting that Mcl-1 is also a substrate for activated caspases.

The caspase-independent decrease of FLIP and Mcl-1 is consistent with the decrease in their expression preceding both cleavage of PARP, a known caspase substrate, and the onset of apoptosis. Furthermore, CHX exposure alone decreased the expression levels of both FLIP and Mcl-1 in the absence of detectable apoptosis. These data suggest that decreased EC levels of FLIP and Mcl-1 following CHX or LPS + CHX exposure are due to intrinsically short half-lives of these proteins and not apoptotic processes.

Proteasome Inhibition Protects against FLIP and Mcl-1 Degradation and LPS + CHX-induced EC Apoptosis—There are two predominant mechanisms for intracellular degradation of proteins, one involving the lysosomal apparatus and the other involving the proteasome (61). The lysosomal pathway is primarily involved in the proteolytic degradation of membrane-bound proteins and extracellular proteins, whereas the protea-
some pathway is primarily responsible for degradation of cytosolic proteins. To determine whether CHX-induced decreases in the levels of FLIP and Mcl-1 could be attributed to rapid turnover via the proteasome, EC were pre-treated with a highly specific proteasome inhibitor, lactacystin, and its derivative β-lactone (62, 63), and subsequently exposed to medium, LPS, CHX, or LPS + CHX (Fig. 3A). Lactacystin protected against decreases in the levels of both FLIP and Mcl-1 could be attributed to rapid turnover via the proteasome, EC were pre-treated with a highly specific proteasome inhibitor, lactacystin, and its derivative β-lactone (62, 63), and subsequently exposed to medium, LPS, CHX, or LPS + CHX (Fig. 3A). Lactacystin protected against decreases in the levels of both FLIP and Mcl-1 in the presence of CHX, indicating that, once protein synthesis is inhibited against decreased levels of both FLIP and Mcl-1 in the presence of CHX (Fig. 3B). EC were pre-treated with lactacystin and subsequently exposed to either TNF-α (10 μg/ml) and its aqueous derivative β-lactone (10 μM) (LAC), and subsequently exposed to either medium or LPS (100 ng/ml) in the presence of CHX (40 μg/ml) for 12 h (A–C). Viability was assayed with the Alamar Blue vital dye (A). Vertical bars represent mean (± S.E.) metabolic activity in arbitrary fluorescent units. *, significantly decreased compared with all other conditions. **, significantly increased compared with LPS + CHX.

FIG. 3. Effect of caspase inhibition on LPS + CHX-induced apoptosis. EC were pre-treated for 1 h with MeSO or the caspase inhibitor peptide, z-VAD (100 μM), and subsequently exposed to medium or LPS (100 ng/ml) in the presence or absence of CHX (40 μg/ml) for 12 h (A–C). Viability was assayed with the Alamar Blue vital dye (A). Vertical bars represent mean (± S.E.) metabolic activity in arbitrary fluorescent units. *, significantly decreased compared with all other conditions. **, significantly increased compared with LPS + CHX. In other experiments, EC lysates were immunoblotted with either an antibody raised against the caspase substrate, PARP (B) or antibodies raised against FLIP or Mcl-1 (C). Molecular mass (in kDa) is indicated.

Proteasome Inhibition Confers Protection against Apoptosis Elicited by TNF-α + CHX or II-1β + CHX—TNF-α and II-1β are established mediators of the acute phase response to Gram-negative bacterial infections (64–66). Similar to LPS, their ability to induce EC apoptosis requires the inhibition of new gene expression (Fig. 5A). To determine whether a common cytoprotective pathway is operative in the resistance of EC to these inflammatory mediators, EC were pre-treated with lactacystin and subsequently exposed to either TNF-α or II-1β in the presence of CHX (Fig. 5B). Lactacystin completely protected against apoptosis elicited by these agents. Interestingly, lactacystin conferred only marginal protection against apoptosis against LPS + CHX-induced apoptosis (data not shown).

The requisite inhibition of new gene expression necessary for EC sensitization to LPS-induced apoptosis suggests that either a constitutive or inducible protein is required for cytoktection. That inhibition of proteasome-dependent protein degradation in the absence of protein synthesis blocks LPS + CHX-evoked apoptosis implicates a cytoprotective role for a constitutively expressed protein(s). In contrast to this finding, a previous study reports that overexpression of an inducible protein, A1, partially protects against LPS + CHX- and TNF-α + CHX-induced EC apoptosis (30, 31). Since the authors report that overexpression of two other cytoprotective proteins, Bcl-xL and A20, also confer partial protection, there is some question as to the physiological implications of these overexpression data. In fact, selective inhibition of A1 gene expression fails to sensitize EC to direct TNF-α killing, suggesting EC sensitization following CHX exposure is not attributed to the inhibition of A1 induction (32).

![Fig. 4. Proteasome inhibition protects against decreased expression of FLIP and Mcl-1 and LPS-induced apoptosis in the absence of new protein synthesis. EC were pre-treated for 30 min with either MeSO or a combination of lactacystin (10 μM) and its aqueous derivative β-lactone (10 μM) (LAC), and subsequently exposed to either medium or LPS (100 ng/ml) in the presence or absence of CHX (40 μg/ml) for 12 h (A and B). EC lysates were immunoblotted with anti-FLIP or anti-Mcl-1 antibodies (A). Molecular mass (in kDa) is indicated. In other experiments, EC viability was measured with Alamar Blue (B). Vertical bars represent mean (± S.E.) metabolic activity in arbitrary fluorescent units. *, significantly decreased compared with all other conditions. **, significantly increased compared with LPS + CHX.](http://www.jbc.org/)
elicited by staurosporine (Fig. 5B), which evokes direct EC killing without requisite inhibition of new gene expression (Fig. 5A). A common signaling pathway shared by LPS, TNF-α, and IL-1β involves the activation of the transcription factor, NF-κB (67). NF-κB regulates the expression of several gene products involved in inflammatory responses, including up-regulation of TNF-α, IL-1β, IL-6, IL-8, VCAM-1, and E-selectin (67, 68). In unstimulated cells, NF-κB is sequestered in the cytoplasm by members of the IκB family. Upon cell activation, IκB is phosphorylated on two serine residues, targeting it for proteasome-mediated degradation. The resultant free NF-κB is then able to translocate to the nucleus, where it promotes new gene expression.

Lactacystin, by virtue of its ability to inhibit proteasome activity, should block NF-κB activation by blocking the degradation of its inhibitor, IκB. As predicted, lactacystin completely blocked LPS-, TNF-α-, and IL-1β-induced up-regulation of VCAM-1, the expression of which is dependent on NF-κB activation (Fig. 6A). These data are in agreement with a previous report that lactacystin inhibits EC NF-κB activation assayed by an electrophoretic mobility shift (69).

The role of NF-κB in apoptosis has been predominantly anti-apoptotic through its ability to up-regulate cytoprotective gene products, including cIAPs (70, 71). Recently, however, a requisite role for NF-κB in promoting apoptosis has been reported (72). Therefore, it was important to rule out that lactacystin was protecting against LPS+CHX-induced apoptosis by blocking NF-κB activation through inhibition of proteasome-mediated degradation of the NF-κB inhibitor, IκB. Using an adenovirus transduction system, EC were transduced with either a
gene for β-galactosidase or 1xBoM with serine to alanine mutations at residues 32 and 36 (14). These serine to alanine mutations prevent signal-induced phosphorylation and proteasome-mediated degradation of the 1xBoM protein (14, 40). LPS-, TNF-α, or IL-1β each up-regulated VCAM-1 expression in non-transduced EC and EC transduced with a control gene, but not in those transduced with the 1xBoM (Fig. 6B). In contrast, 1xBoM transduction did not affect the sensitivity of EC to LPS-, TNF-α, or IL-1β-induced killing at 12 h in the absence of de novo protein synthesis (Fig. 6C). These data suggest that the protective effect elicited by lactacystin is through inhibition of the degradation of a cytoprotective protein, and not through the blockade of a pro-apoptotic NF-κB signaling pathway. Further, the presumed mechanism of the pro-apoptotic properties of NF-κB reported by Ryan et al. (72) is through the promotion of new gene expression, similar to its anti-apoptotic role and the induction of cIAPs. The finding that LPS, TNF-α, and IL-1β all stimulate EC apoptosis in the absence of new protein synthesis argues against a pro-apoptotic role for NF-κB involving new gene expression. Finally, transduction of EC with the 1xBoM gene sensitizes these cells to apoptosis induced by long term exposure (≥24 h) to TNF-α, implicating an anti-apoptotic role for NF-κB and not a pro-apoptotic role (14).

**FLIP Antisense Sensitizes EC to LPS-induced Apoptosis at Low CHX Concentration—**To elucidate a definitive anti-apoptotic role for either FLIP and/or Mcl-1 against LPS-induced apoptosis, antisense oligonucleotides were designed to specifically reduce the expression of each protein. Western blot analysis of EC transfected with either FLIP or Mcl-1 antisense revealed a dramatic decrement in the expression of FLIP and Mcl-1, respectively, compared with EC transfected with mismatch oligonucleotide controls (Fig. 7, A and C). Under identical conditions, we exposed the transfected cells to medium or LPS (100 ng/ml) for 12 h and assayed for histone release (Fig. 7, B and D). The decrease in FLIP or Mcl-1 with antisense oligonucleotides failed to directly sensitize EC to LPS-induced apoptosis. Since the antisense oligonucleotides reduced the level of each protein but failed to completely ablate expression, we postulated that minimal threshold level expression of either protein could confer protection. To test this hypothesis, we exposed EC transfected with FLIP or Mcl-1 antisense to LPS and a low concentration of CHX (2 μg/ml) and assayed for protein expression levels (Fig. 7, A and C) and apoptosis (Fig. 7, B and D). FLIP antisense in combination with low dose CHX (2 μg/ml) resulted in almost complete knockout of FLIP expression (Fig. 7A) and sensitized EC to LPS killing (Fig. 7B). In contrast, EC transfected with mismatch control oligonucleotides had detectable levels of FLIP expression and were resistant to LPS killing. Doubling the concentration of CHX sensitized these cells to LPS killing, albeit to a lesser extent than those transfected with FLIP antisense (Fig. 7B). These data implicate a role for the constitutively expressed protein FLIP in conferring protection against LPS-induced EC apoptosis. Using
a low dose of CHX in combination with FLIP antisense, cellular levels of FLIP were reduced to barely detectable limits, and sensitivity to LPS killing was conferred. Importantly, this concentration of CHX alone failed to sensitize control EC to LPS killing, indicating that the specific reduction of FLIP sensitized EC to LPS-induced apoptosis. FLIP levels in control-transfected EC exposed to low dose CHX (2 μg/ml) were comparable to those in EC transfected with FLIP antisense without CHX exposure (Fig. 7A). In both cases, sensitivity to LPS killing was not observed, suggesting that a threshold level of FLIP conferred cytoprotection. At higher concentrations of CHX, a dose-dependent increase in apoptosis among control-transfected EC was demonstrated (Fig. 7B), consistent with Fig. 2, in which high concentrations of CHX completely diminished FLIP levels. Although this finding clearly implicates a cytoprotection role for FLIP, it does not preclude the requisite involvement of another unidentified cytoprotective protein. It is possible that sensitivity to LPS killing was conferred to EC transfected with FLIP antisense, not only by reducing FLIP expression below minimal threshold levels necessary for protection, but also by reducing the level of another cytoprotective protein.

Similar to FLIP antisense, Mcl-1 antisense, in the presence of low dose CHX (2 μg/ml) decreased Mcl-1 expression below detectable levels (Fig. 7C). However, in contrast to FLIP antisense, Mcl-1 antisense neither sensitized EC to LPS-induced apoptosis nor enhanced killing compared with mismatch oligonucleotides in the presence of any concentration of CHX tested (Fig. 7D).

In summary, we have characterized a cytoprotective pathway in EC that confers resistance against LPS-induced apoptosis (Fig. 8). First, we established that inhibition of gene expression sensitizes EC to LPS-evoked killing by blocking de novo synthesis of a constitutively expressed cytoprotective protein(s), not an inducible protein. Second, this cytoprotective protein(s) has a relatively short half-life and is degraded through the proteasome. Third, EC apoptosis induced by two other inflammatory mediators, TNF-α and IL-1β, in combination with a protein synthesis inhibitor is blocked by proteasome inhibition, suggesting a common cytoprotective pathway dependent upon constitutive expression of an anti-apoptotic protein(s). Fourth, by screening EC for known constitutively expressed anti-apoptotic proteins, the expression of which is acutely sensitive to inhibition of new gene expression and proteasome degradation, we identified a potential cytoprotective role against LPS-induced apoptosis for two proteins, FLIP and Mcl-1. Finally, using antisense oligonucleotides, we have identified a definitive role for FLIP in conferring protection against LPS-induced apoptosis in human EC.

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J. Biol. Chem. 2001, 276:14924-14932.
doi: 10.1074/jbc.M100819200 originally published online February 14, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100819200

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