Mechanisms of bacterial lipopolysaccharide-induced endothelial apoptosis

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Bannerman, Douglas D., and Simeon E. Goldblum. Mechanisms of bacterial lipopolysaccharide-induced endothelial apoptosis. Am J Physiol Lung Cell Mol Physiol 284: L899–L914, 2003; 10.1152/ajplung.00338.2002.—Gram-negative bacterial sepsis remains a common, life-threatening event. The prognosis for patients who develop sepsis-related complications, including the development of acute respiratory distress syndrome (ARDS), remains poor. A common finding among patients and experimental animals with sepsis and ARDS is endothelial injury and/or dysfunction. A component of the outer membrane of gram-negative bacteria, lipopolysaccharide (LPS) or endotoxin, has been implicated in the pathogenesis of much of the endothelial cell injury and/or dysfunction associated with these disease states. LPS is a highly proinflammatory molecule that elicits a wide array of endothelial responses, including the upregulation of cytokines, adhesion molecules, and tissue factor. In addition to activation, LPS induces endothelial cell death that is apoptotic in nature. This review summarizes the evidence for LPS-induced vascular endothelial injury and examines the molecular signaling pathways that activate and inhibit LPS-induced endothelial apoptosis. Furthermore, the role of apoptotic signaling molecules in mediating LPS-induced activation of endothelial cells will be considered.

endotoxin; inflammation; nuclear factor-κB; sepsis; vascular injury

DESPITE ADVANCES IN ANTIMICROBIAL therapy and overall medical care, gram-negative bacterial sepsis remains a common, life-threatening event (163). Complications arising from sepsis include disseminated intravascular coagulation (133, 197, 201), systemic vascular collapse (88, 201), multiorgan failure (22, 23, 65), and the development of vascular leak syndromes, including acute respiratory distress syndrome (ARDS) (26, 27, 142, 144, 161). One common denominator to all of these complications is endothelial cell (EC) injury and/or dysfunction (46, 47, 80, 132, 148, 153).

The vascular endothelium serves as the key barrier between the intravascular compartment and extravascular tissues and plays a critical role in a large number of physiological and pathological processes (38). ECs are integrally involved in regulating blood flow, coagulation, leukocyte trafficking, edema formation, wound healing, and angiogenesis. Because of their location at the blood-extravascular tissue interface, ECs are constantly exposed to circulating mediators that may perturb the above-mentioned endothelial barrier functions. One such mediator that targets the endothelium is bacterial lipopolysaccharide (LPS) or endotoxin, a highly proinflammatory molecule that is a component of the outer envelope of all gram-negative bacteria (17, 159, 176). LPS is released from the surface of replicating and dying gram-negative bacteria into the circulation, where it interacts with the endothelial lining of the vessel wall (173, 176). Evidence exists that LPS, alone or in concert with other endogenous factors, is responsible for much of the EC injury and/or dysfunction associated with gram-negative sepsis. First, LPS bioactivity has been detected in the bloodstream of gram-negative septicemic patients, and in selected studies, levels of circulating LPS predict the development of multiorgan failure, including ARDS (26). Second, administration of LPS alone to experimental animals reconstitutes the EC injury seen after gram-negative bacterial challenge (27, 201). Third, immunological and pharmacological interventions that specifically target the LPS molecule protect against these same vascular complications (6, 210, 211, 225). Finally, LPS directly elicits several of the EC responses in vitro that

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are similarly evoked during sepsis, including: 1) the production of the proinflammatory cytokines IL-6 (77, 109), IL-8 (7, 77, 223), and IL-1β (57, 138); 2) the increased surface expression of the adhesion molecules E-selectin, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 (34, 59, 77, 108); and 3) increased expression of tissue factor (42, 75). In addition to activation, LPS induces EC-programmed cell death or apoptosis (18, 21, 64, 67, 82, 95, 116, 143, 221), an event that is believed to contribute to the pathogenesis of sepsis and its attendant complications (82, 189). Several of the signaling molecules involved in EC activation leading to increased cytokine and adhesion molecule expression are similarly involved in LPS proapoptotic signaling. Furthermore, there is evidence that LPS proapoptotic signaling molecules have an additional role in regulating LPS-induced NF-κB activation and nonapoptotic EC responses to LPS. This review focuses on recent advances in the elucidation of the mechanisms by which LPS elicits EC apoptosis and the cross talk between signaling pathways leading to EC activation and death.

EVIDENCE FOR LPS-INDUCED EC APOPTOSIS

Apoptosis is an ATP-dependent form of cell death, morphologically characterized by chromatin condensation, nuclear fragmentation, cell shrinkage, and blebbing of the plasma membrane (76, 171). The end result of apoptosis is fragmentation of the cell into small membrane-bound bodies that are quickly cleared by phagocytic cells (45, 181, 187). Biochemically, apoptosis is characterized by the activation of caspases, highly specific proteases that cleave a wide array of intracellular substrates (198). Activation of upstream caspases initiates a proteolytic cascade leading to DNA fragmentation and the cleavage of key regulatory proteins resulting in cell death. In contrast to apoptosis, necrotic cell death is an energy-independent process characterized by cell swelling and lysis (76, 171). Unlike apoptotic cells, necrotic cells release cellular constituents that elicit an inflammatory reaction in surrounding viable tissue.

LPS induces apoptosis in bovine and ovine ECs in vitro (18, 64, 94, 95) and elicits human EC apoptosis in the absence of new gene expression (164). That LPS-induced EC death is apoptotic in nature has been confirmed by several criteria, including morphological changes (64), DNA laddering (18, 99), TdT-mediated dUTP nick end labeling (18, 94, 95), nuclear histone release (15, 21), caspase activation (15, 19), and poly-(ADP-ribose)polymerase cleavage (21, 36). Several studies have demonstrated that purified LPS itself, in the absence of host-derived mediators, evokes EC injury and/or apoptosis (18, 36, 64, 67, 82, 95, 116, 221). The ability of LPS to induce EC apoptosis in the absence of non-EC-derived host mediators is compatible with a direct effect. Furthermore, neutralizing antibodies to other known death receptors expressed on EC, including TNF type 1 receptor (TNFR1), Fas, and death receptor 3 (DR3), fails to inhibit LPS-induced apoptosis (36). Finally, LPS-induced EC apoptosis occurs independently of new protein synthesis, thereby precluding the involvement of upregulated gene products (84).

CLINICAL RELEVANCE AND IN VIVO EVIDENCE

Endothelial apoptosis has been implicated in the pathogenesis of several disease states, including atherosclerosis (30), hypertension (73), congestive heart failure (204), and systemic capillary leak syndrome (12). Several studies have reported that EC injury and/or death is a key pathological finding during bacterial sepsis. First, injection of *Escherichia coli* into rabbits (47) or baboons (40, 41) induces severe microvascular injury and EC detachment. Second, increased numbers of apoptotic ECs are detected in the pulmonary capillaries of a murine model of sepsis (93, 217). Third, evidence of EC injury is observed in postmortem biopsies obtained from patients who have died of sepsis-related ARDS (148). Fourth, an increase in circulating EC is observed in septic patients, and the magnitude of this increase correlates negatively with survival (153).

There are several reports that purified LPS elicits EC injury and apoptosis in vivo. EC injury and/or detachment from the vascular wall has been reported after LPS injection into mice (124), rats (172, 192), rabbits (47, 69, 145), dogs (72, 127), sheep (27, 149), and primates (14). Liver sinusoidal ECs obtained from LPS-treated rats display enhanced activation of caspase-3, a central apoptotic effector protease (49). Disseminated EC apoptosis has been reported in the lung, liver, thymus, and intestine of mice challenged with LPS (67, 82, 116, 122). Finally, injection of a broad spectrum caspase inhibitor decreases EC apoptosis in the lung after LPS administration and improves survival in a murine model of acute lung injury (116). The combined in vitro and in vivo data implicate apoptosis as a key component of the EC response to LPS. However, the extent to which LPS directly induces EC apoptosis in human systems either alone or in concert with other known apoptosis-inducing proinflammatory cytokines, such as TNF-α and/or IL-1β, remains unclear.

CELL SURFACE RECOGNITION OF LPS

As the name implies, LPS is composed of both lipid and polysaccharide components. The lipid portion is composed of a unique lipid, lipid A, which is the most widely conserved region of the LPS molecule (174). It is well established that the lipid A moiety of LPS is responsible for its proinflammatory properties (174). Lipid A alone is capable of eliciting EC responses identical to those induced by LPS (16, 50, 193). Agents that specifically target the lipid A moiety of LPS inhibit EC activation (16, 52) and protect against the development of vascular complications in endotoxin shock models (6, 210). Similar to activation, the lipid A moiety is also responsible for the proapoptotic properties of
the LPS molecule (84). Neutralization of lipid A with polymyxin B completely abrogates LPS-induced EC apoptosis (18, 19, 64).

An early identified receptor implicated in cellular recognition of LPS was membrane-associated CD14 (mCD14) (202). mCD14 is a glycoprotein found on cells of monocytic origin and to a lesser extent on neutrophils. Although LPS can directly bind mCD14, its affinity for the receptor is greatly increased when LPS is complexed with the acute phase protein LPS-binding protein (LBP) (159). ECs, which lack mCD14, are activated by LPS in the presence of LBP and soluble CD14 (sCD14), the latter of which is released from mCD14-bearing cells (9, 74). It has been proposed that LBP facilitates the transfer of LPS to sCD14 and that this LPS-sCD14 complex is recognized by ECs (202, 205). Similar to EC activation, a requisite role for sCD14 in mediating LPS-induced apoptosis has been established. In the absence of sCD14, ECs are resistant to LPS-induced apoptosis (9, 64, 219). In the presence of CD14-containing serum, anti-CD14 antibodies inhibit LPS-induced EC cytotoxicity (9, 64, 219). Because CD14 is a glycosyl phosphatidylinositol-anchored protein and lacks an intracellular cytoplasmic domain, it was postulated that another transmembrane receptor must exist that can transduce LPS signaling across the plasma membrane. This receptor, which has since been identified as Toll-like receptor (Tlr)-4, is expressed in cells of monocytic lineage as well as in non-mCD14-bearing ECs (37, 61). There has been some controversy in the past regarding whether another member of the Toll-like receptor family, Tlr-2, mediates LPS-induced signaling. Two groups have reported that LPS-induced activation is mediated by Tlr-2 (121, 218). However, a subsequent study reported that certain commercial preparations of LPS are contaminated with bacterial lipoproteins and that these lipoproteins, not LPS, are responsible for Tlr-2 activation (90). The genetic evidence clearly establishes Tlr-4 as a true LPS receptor (166). C3H/HeJ and C57BL/10ScCr mice, which contain a missense mutation in or a null mutation for the Tlr-4 gene, respectively, are resistant to LPS (166). Furthermore, transfection of Tlr-4 into LPS-insensitive HEK-293 cells confers sensitivity to LPS-induced NF-κB activation (37). Finally, anti-Tlr-4 antibodies abrogate LPS-induced EC NF-κB activation, whereas anti-Tlr-2 antibodies have no inhibitory effect (61).

**TLR-4 SIGNALING LEADING TO NF-κB ACTIVATION**

Tlr-4 is integrally involved in LPS signaling and has a requisite role in the activation of the transcription factor NF-κB. The extracellular domain of Tlr-4 contains repeating leucine-rich motifs characteristic of innate immune response pattern recognition receptors (Fig. 1) (48). The cytoplasmic domain contains regions that are homologous to the intracellular signaling domain of the type 1 IL-1 receptor. Although the exact mechanism by which LPS is recognized by Tlr-4 remains unclear, cell activation is dependent on the cell surface assembly of a multiprotein recognition complex consisting of CD14, MD-2, and Tlr-4 (3) (Fig. 2). After activation of the Tlr-4 receptor complex, the adapter protein myeloid differentiation factor 88 (MyD88) is
recruited to the cytoplasmic domain of Tlr-4 through homotypic binding of respective Toll receptor-IL-1 receptor (TIR) domains (146, 154). MyD88 contains an additional protein-binding domain, the death domain (DD) (Fig. 1), which facilitates its association with another DD-containing signaling molecule, IL-1 receptor-associated kinase-1 (IRAK-1) (48). After autophosphorylation, IRAK-1 dissociates from MyD88 and interacts with TNF receptor-associated factor-6 (TRAF-6) (137, 194), resulting in the activation of a downstream kinase cascade involving NF-κB-inducing kinase (NIK) and IκB kinase (IKK) (Fig. 2). The IKK-mediated phosphorylation of IκB, an inhibitor of NF-κB, leads to IκB degradation through the proteasome pathway and enables NF-κB to translocate to the nucleus where it promotes new gene expression (48).

**TLR-4 SIGNALING LEADING TO APOPTOSIS**

Several of the upstream signaling molecules involved in LPS activation of NF-κB are similarly involved in promoting LPS-induced apoptosis. Macrophages derived from C3H/HeJ mice, which have a missense mutation in the third exon of Tlr-4 (166), are resistant to LPS-induced apoptosis (110). Furthermore, neutralization of the lipid A moiety of LPS, which is the domain of LPS recognized by Tlr-4 (139), protects against LPS-induced EC apoptosis (18, 19). Downstream of Tlr-4, MyD88 and IRAK-1 have been implicated in mediating LPS-elicited cell death signaling. Expression of either MyD88 or IRAK-1 dominant-negative (D/N) constructs, which inhibit LPS-induced NF-κB activation (19, 222), protects against LPS-induced EC apoptosis (19). MyD88 has similarly been shown to mediate Tlr-2 activation of NF-κB and proapoptotic signaling (5). Downstream of MyD88 and IRAK-1, TRAF-6 has been implicated in promoting LPS-induced EC apoptosis (100). TRAF-6 participation in LPS-induced apoptosis involves c-jun NH2-terminal kinase, the activation of which lies upstream of caspase activation. Together, these data indicate that Tlr-4 can serve as a death receptor for LPS and that the signaling molecules involved in LPS-induced NF-κB activation serve a dual role in promoting LPS-induced apoptosis (Fig. 2).

There is evidence suggesting that Tlr-4 activation of NF-κB can occur through a MyD88- and IRAK-1-independent pathway. The LPS-induced DNA-binding activity of NF-κB in macrophages derived from either MyD88 or IRAK-1 knockout mice is delayed, but not inhibited, indicating that cellular activation by LPS can occur in the absence of these signaling molecules (115, 194). Recently, an MyD88-like protein has been identified by two independent groups, MyD88 adapter-like protein (MAL) or TIR domain-containing adapter protein (TIRAP), which promote LPS-induced NF-κB signaling through IRAK-2 (62, 91). MAL/TIRAP contains a COOH terminus TIR domain but lacks the NH2 terminus DD present in MyD88 (Fig. 1). Similar to MyD88, MAL/TIRAP has an additional role in promoting LPS-induced EC apoptosis (15). These studies demonstrate the presence of an MyD88-independent pathway that serves a redundant signaling role in promoting both LPS-induced NF-κB activation and apoptosis in ECs (Fig. 2).

Although Tlr-4 and its respective intracellular binding partners MyD88 and MAL/TIRAP have been shown to mediate LPS-induced apoptosis, questions remain as to how this signaling pathway activates the effector proteases of apoptosis, the caspases. The Fas-associated death domain (FADD) is a proapoptotic adapter protein that couples death receptors to initiator caspases (24, 35, 96, 126). Its role in mediating apoptosis has been well elucidated in death signaling initiated by the Fas and TNF-α receptors. FADD recruitment, either directly to Fas or through the intermediary TNF receptor-associated DD protein in the case of the TNF-α receptor, is mediated through binding between the two highly conserved DDs found on these proteins. Similarly, FADD and procaspase-8 interact through the death effector domains (DED) contained by each protein (Fig. 1). Procaspase-8 contains partial proteolytic activity that enables it to cleave other procaspase-8 molecules brought into close proximity after recruitment to FADD (155). Activation of caspase-8 initiates a proteolytic cascade resulting in the activation of downstream effector caspases, including caspase-3 (89). Although a role for FADD in mediating TNF-α- and Fas-induced apoptosis has been well established, there are conflicting reports as to whether this molecule is involved in LPS proapoptotic signaling. Choi et al. (36) reported that expression of a FADD D/N protected against LPS-induced EC apoptosis, whereas another group showed that the same FADD D/N had no effect (19). It is difficult to reconcile these differences because both studies utilized the same EC type and a comparable retrovirus-based system to stably express identical FADD D/N constructs. Furthermore, both studies were able to demonstrate the efficacy of the FADD D/N construct in protecting against TNF-α-elicited apoptosis. One major difference between the studies was in the assay used to measure cell death. In the study by Choi et al. (36), mitochondrial activity was used as a measure of cell viability. This method of viability, however, does not discriminate between necrotic and apoptotic cell death. In the contrasting report, a more selective assay for determining apoptotic cell death, which involved measuring caspase activity, was used (19). One explanation for these differences may be that LPS induces both apoptotic (caspase-dependent) and nonapoptotic (caspase-independent) cell death and that FADD plays a role in mediating the latter.

Tlr-4 is one member of a larger class of pattern recognition receptors involved in the innate immune response. Another member of the Tlr family, Tlr-2, recognizes the cell wall components peptidoglycan and bacterial lipoproteins found in both gram-positive and -negative bacteria as well as gram-positive-restricted lipoteichoic acid (4, 186). Several of the intracellular signal transduction molecules involved in Tlr-4-mediated activation of NF-κB, including MyD88, IRAK,
TRAF-6, NIK, and IKK, are similarly involved in Tlr-2 signaling. Interestingly, bacterial lipoprotein-elicited Tlr-2 proapoptotic signaling is reportedly mediated by MyD88 and FADD, two molecules similarly involved in LPS/Tlr-4-induced apoptosis (5). In contrast, Tlr-2-mediated apoptosis occurs independently of TRAF-6 involvement (5), a signaling molecule recently established to participate in LPS/Tlr-4 proapoptotic signaling (100). Thus divergence in the apoptotic signaling pathways mediated by Tlr-2 and Tlr-4 occurs downstream of MyD88.

**ROLE OF NF-κB ACTIVATION IN MEDIATING LPS-INDUCED APOPTOSIS**

LPS-induced activation of NF-κB is a key signaling event that mediates an array of EC responses, including increased 1) IL-6 (109), IL-8 (7, 225), and IL-1β (138) production, 2) E-selectin, ICAM-1, and VCAM-1 surface expression (34, 108), and 3) tissue factor activity (42, 75). In addition to its role in promoting the expression of proinflammatory gene products, NF-κB has been implicated in both pro- (86, 178) and antiapoptotic signaling (128, 203). In ECs, inhibition of NF-κB activation sensitizes human ECs to direct TNF-α-induced apoptosis in the absence of cycloheximide, suggesting an antiapoptotic role for NF-κB (221). Evidence exists that this sensitization is conferred by inhibition of NF-κB-dependent expression of members of the inhibitors of apoptosis (IAP) gene family (190). In contrast to TNF-α, inhibition of NF-κB does not sensitize human ECs to direct LPS-induced apoptosis (221). These data, therefore, preclude an antiapoptotic role for NF-κB in conferring protection against LPS-elicted apoptosis.

There are reports that NF-κB signaling has a role in proapoptotic signaling (86, 178). On the basis of the predominant role of NF-κB in mediating LPS-elicted EC responses and the finding that MyD88, MAL/TTRAP, and IRAK-1 promote both LPS-induced NF-κB activation and apoptosis (Fig. 2), there has been speculation that these events are coupled. Several lines of evidence, however, suggest that these events occur independently of one another. First, the presumed mechanism of the proapoptotic properties of NF-κB reported by Ryan et al. (178) is through the promotion of new gene expression, similar to its antiapoptotic role and the induction of antiapoptotic proteins. The finding that LPS elicits EC apoptosis in the absence of new protein synthesis argues against a proapoptotic role for NF-κB that involves new gene expression (21). Second, inhibition of NF-κB activation by overexpression of an IκBα superrepressor fails to block LPS-induced EC apoptosis (19, 21). This latter finding also demonstrates a bifurcation in the NF-κB and apoptotic pathways that mediate LPS signaling upstream of IκB degradation. Finally, complete inhibition of apoptosis with the caspase inhibitor zVAD-FMK does not inhibit the ability of LPS to activate NF-κB (19). Thus despite a commonality among upstream signaling molecules, LPS/Tlr-4-induced NF-κB activation and apoptosis are mutually independent events (Fig. 2).

**ROLE OF CASPASES**

A hallmark of apoptosis is the activation of highly specific effector proteases of the caspase family. Caspases exist as inactivezymogens(proenzymes) that are activated by proteolytic processing of the pro-caspase molecule in one of three ways: 1) autoactivation due to low levels of intrinsic catalytic activity, 2) transactivation by other caspases within close proximity, or 3) activation by noncaspase proteases (198). Once activated, caspases cleave and activate other members of the caspase family leading to amplification of a proteolytic cascade. The end result is a series of proteolytic events that lead to the cleavage of intracellular substrates, chromatin condensation, DNA fragmentation, and eventual cell death.

Of the 14 known caspases expressed in mammalian cells, LPS has been reported to activate caspase-1 (152, 185), caspase-3 (15, 49, 100, 111, 152), caspase-6 (111), and caspase-8 (100, 111). Activation of caspase-1 (interleukin-1 converting enzyme) is primarily responsible for IL-1β processing and has limited involvement in apoptosis (43). In contrast, caspase-3, -6, and -8 all have been established to prominently participate in apoptosis. Caspase-8, also known as FADD-like interleukin converting enzyme protease (FLICE), is classically described as an initiator caspase that is recruited to such death receptors as TNFR and Fas. Procaspase-8 has intrinsically low levels of proteolytic activity that enables it to cleave other procaspase-8 molecules brought into close proximity after recruitment to the receptor (155). Activation of caspase-8 initiates a proteolytic cascade resulting in the activation of downstream effector caspases and the onset of apoptosis (89). A key target of caspase-8 is procaspase-3. Proteolytic processing of procaspase-3 results in an active caspase-3 molecule that serves as a key effector caspase responsible for much of the proteolysis associated with apoptosis (89, 107). The effects of LPS-induced caspase activation on ECs are quite dramatic, resulting in the cleavage of nuclear proteins as well as structural proteins that mediate cell-cell and cell-substrate adhesion (18, 21). In vivo studies support a role for caspases in mediating LPS-induced EC apoptosis (49, 116). Liver ECs derived from mice administered intravenous LPS display enhanced caspase-3 activity (49). Furthermore, inhibition of caspase activation with the cell-permeable, pan-caspase inhibitor peptide zVAD protects against EC apoptosis elicited by LPS in a murine model of endotoxin shock (116).

The mechanism by which LPS/Tlr-4 signaling activates caspases remains unknown. A proximal event in LPS signaling is MyD88 recruitment of IRAK through reciprocal binding of the DD regions on each of these molecules (48). One possibility is that FADD or a FADD-like protein may bind either MyD88 and/or IRAK-1 through homophilic DD-DD interactions (Fig. 2). By a mechanism similar to that reported for other
death receptor signaling pathways, including those involving Fas and TNFR1 (11), FADD, which contains an additional DED binding domain, may recruit procaspase-8 via DED-DED interactions to the Tlr-4 signaling complex. The induced proximity of procaspase-8 molecules could lead to respective activation and the onset of apoptosis. In support of this hypothesis, overexpressed MyD88 has been reported to bind FADD via DD-mediated interactions (5, 92). Whether this interaction occurs when these proteins are expressed at physiological levels remains unknown.

ANTI-APOPTOTIC SIGNALING AND ROLE OF FLICE-LIKE INHIBITOR PROTEIN

FLICE-like inhibitor protein (FLIP) is an antiapoptotic protein with significant homology to caspase-8 (FLICE) (106). In FLIP, a substitution of two amino acids that corresponds to the caspase-8 catalytic site renders it catalytically inactive. The role of FLIP in the inhibition of Fas death signaling has been well elucidated. On Fas ligand binding, the adapter protein FADD is recruited to the Fas receptor via an interaction between the DD of each protein (199). FLIP and procaspase-8 are, in turn, recruited to FADD via DED protein-protein interactions contained within all three proteins (Fig. 1). It has been proposed that FLIP can inhibit activation of upstream initiator caspases, including caspase-8, by competitively binding to FADD and blocking assembly of a functional death signaling complex (188). Alternatively, FADD recruitment of FLIP, which lacks intrinsic proteolytic activity, may prevent transactivation of procaspase-8 (182). A role for FLIP in inhibiting TNFR1-elicited apoptosis through a similar mechanism has been reported (97).

Recent studies indicate that FLIP protects against LPS-induced apoptosis. First, decreased expression of FLIP parallels in both a dose- and time-dependent manner with EC sensitization to LPS-induced apoptosis (21, 60). Second, inhibition of proteasome-mediated FLIP degradation protects against LPS-induced apoptosis (21, 60). This suggests that cycloheximide or Shiga-like toxin sensitizes ECs to LPS-induced apoptosis. Recently, inhibition of proteasome-mediated protein degradation was shown to protect against LPS-induced apoptosis in the presence of cycloheximide (21). That LPS-evoked apoptosis was prevented in the absence of new gene expression precludes a role for an inducible gene product. Interestingly, inhibition of de novo protein synthesis leads to a marked decrease in the expression of FLIP, a constitutively expressed protein implicated in conferring resistance to LPS-induced apoptosis (21, 60). This suggests that cycloheximide sensitization of human ECs to LPS-induced apoptosis is mediated by both the inhibition of de novo synthesis of FLIP and the rapid degradation of preexisting FLIP molecules via the proteasome (Fig. 3).

Fig. 3. Schematic diagram of the mechanism by which cycloheximide (CHX) or Shiga-like toxin sensitizes ECs to LPS-induced apoptosis. Under physiological conditions, constitutive expression of FLIP confers resistance to LPS-induced apoptosis in human ECs. In the presence of CHX or Shiga-like toxin (SLT), de novo synthesis of FLIP is inhibited. Preexisting molecules of FLIP are rapidly degraded via the proteasome. The end result of CHX or SLT exposure is diminished expression of FLIP and sensitization of human ECs to LPS-induced apoptosis.
Although cycloheximide or actinomycin D have been used as tools to investigate sensitization to LPS-induced apoptosis by inhibiting de novo gene expression, it is difficult to imagine a scenario in which these agents would be present in vivo with LPS. However, naturally occurring agents that inhibit protein synthesis may be expected to confer similar sensitivity. Shiga toxin and Shiga-like toxin-1 (SLT-1), produced by *Shigella dysenteriae* serotype 1 and certain strains of *E. coli*, respectively, inhibit protein synthesis (141, 162, 213). Both of these toxins have been implicated in the pathogenesis of hemolytic uremic syndrome and its attendant EC injury (158, 167, 170). Shiga toxin and SLT-1 inhibit protein synthesis by cleaving a specific bond in the 28S rRNA component of the 60S ribosomal subunit, resulting in the release of a single adenine base and the inhibition of aminoacyl tRNA binding to the ribosome (79, 158). Recent findings suggest that SLT-1 sensitizes human ECs to LPS-induced apoptosis by virtue of its protein synthesis inhibitory properties (60) (Fig. 3). SLT-1 inhibition of FLIP expression sensitizes ECs to LPS-induced apoptosis in both a dose- and time-dependent manner. Furthermore, sustained expression of FLIP in the presence of SLT-1 abrogates SLT-induced sensitization of human ECs to LPS-induced apoptosis. Thus strains of *E. coli* that produce SLT-1 have all the necessary components to elicit EC apoptosis, namely a sensitizing agent, SLT-1, and an inducer of apoptosis, LPS. Other naturally occurring agents reported to have protein synthesis inhibitory properties include *Pseudomonas aeruginosa* toxin (101), interferons (39), and TNF-α (129). The role of these agents in sensitizing ECs to LPS-induced apoptosis during mixed infection and inflammation should be considered. Furthermore, patients in severe sepsis often are in a profound catabolic state (85). Whether this influences the ability of cells to maintain adequate levels of antiapoptotic proteins while being exposed to LPS and/or apoptotic-inducing cytokines remains unknown.

**ROLE OF BCL-2 FAMILY MEMBERS**

Cell commitment to apoptosis is governed by both pro- and antiapoptotic signaling pathways. The Bcl-2 family of proteins plays a central role in mediating these two opposed pathways (2, 8). The Bcl-2 family is composed of both pro- and antiapoptotic members. A key mechanism by which these proteins mediate apoptosis is through the regulation of cytochrome c release from the mitochondrion (2, 8). Cytochrome c is a cofactor in the activation of caspase-9, the latter of which activates downstream effector caspases, including caspase-3. Proapoptotic and antiapoptotic members of the Bcl-2 family facilitate and restrict, respectively, cytochrome c release from the mitochondrion. The various Bcl-2 family members are characterized by the presence of one or more of four distinct Bcl-2 homology (BH) domains that facilitate protein-protein interactions. Antiapoptotic Bcl-2 family members exert their effect by forming heterodimers via these BH domains with the proapoptotic members, thereby impairing the ability of the proapoptotic members to induce cytochrome c release.

Several lines of evidence suggest that members of the Bcl-2 family mediate sepsis- and LPS-induced apoptosis. First, in a murine model of sepsis induced by cecal ligation and puncture, enhanced endothelial apoptosis parallels a decrement in the expression of the antiapoptotic protein Bcl-2 (217). Second, LPS upregulates EC expression of the proapoptotic Bcl-2 family members Bax (81, 118, 152), Bad (147), and Bak (147). LPS has also been reported to upregulate expression of A1 (99) and to downregulate levels of Bcl-2 (81, 118) and Bcl-xL (147), all of which are antiapoptotic members of the Bcl-2 family. Third, vascular endothelial growth factor (VEGF) (152) and other agents (81, 118) that inhibit LPS-induced upregulation of proapoptotic Bcl-2 family members protect against LPS-induced EC apoptosis. Inhibition of LPS-induced downregulation of antiapoptotic Bcl-2 homologs similarly confers protection (81, 118). Finally, overexpression of A1 or Bcl-xL protects against LPS-induced EC apoptosis (99). Together, these data implicate a role for Bcl-2 homologs in determining EC fate after LPS exposure.

**ROLE OF NITRIC OXIDE**

Nitric oxide (NO) is a biological messenger molecule with profound influence on the vasculature. NO has been implicated in the regulation of vasomotor tone, inhibition of platelet aggregation and leukocyte adhesion to ECs, activation of transcription factors leading to new gene expression, and the regulation of apoptosis (140). In ECs, NO is generated by both a constitutive nitric oxide synthase and an inducible nitric oxide synthase (iNOS) (168). LPS upregulates the expression of iNOS and the production of NO in ECs (130, 156, 175, 179). These EC responses are further potentiated by LPS coadministration with other proinflammatory cytokines, including interferon-γ (70, 208).

NO has been reported to inhibit LPS-induced EC apoptosis. ECs exposed to the NO donors S-nitroso-N-acetylpenicillamine or (Z)-1-[2-(2-aminoethyl)-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate demonstrate reduced sensitivity to LPS-induced apoptosis (51, 196). Enhanced production of endogenous NO after overexpression of iNOS has been similarly shown to block LPS-elicited EC apoptosis (31, 200). The mechanism by which NO inhibits LPS-induced EC apoptosis remains unclear. Increased levels of NO have been reported to suppress LPS-induced activation of caspase-3 in ECs (31, 200). In vitro studies using purified human recombinant caspases have shown that NO reversibly inhibits seven distinct members of the caspase family, including caspase-3, through direct S-nitrosylation of a cysteine residue required for caspase catalytic activity (55, 119, 136). Thus the protective effect of NO on LPS-exposed ECs may be mediated by NO inactivation of caspases.

In contrast to a cytoprotective role for NO, other studies have reported that suppression of iNOS induction and inhibitors of NOS activity protect against
LPS-elicited EC injury (87, 160, 180), suggesting that NO promotes LPS-induced apoptosis. The differential effects of NO on mediating LPS-induced EC apoptosis appear to be dose dependent. DeMeester et al. (51) have reported that moderate levels of NO confer protection, whereas higher concentrations of NO enhance LPS-induced EC apoptosis. Although the mechanism by which NO potentiates LPS-elicited apoptosis remains unknown, high concentrations of NO can inhibit protein synthesis (214). Because protein synthesis inhibition has been established to sensitize human ECs to LPS-induced apoptosis by inhibiting the expression of the antiapoptotic protein FLIP (21), one may speculate that high levels of NO may result in decreased expression of this cytoprotective protein. Alternatively, increased production of NO after LPS induction of iNOS may result in NO reaction with superoxide anion to form peroxynitrite. EC injury resulting from the generation of peroxynitrite, a potent oxidizer, may synergistically enhance the EC apoptosis elicited by LPS. Further studies are needed to clarify the potential proapoptotic role of NO in mediating LPS-induced EC apoptosis.

ROLE OF REACTIVE OXYGEN SPECIES

In response to stress and/or injury, ECs generate reactive oxygen species (ROS) and nitrogen intermediates (165). Generation of these free radicals can lead to an alteration in the balance of the pro- and antioxidant states, resulting in oxidative stress and potential cell injury (32). Increases in ROS or depletion of antioxidants can lead to apoptosis in a variety of cell types, including ECs (54, 68, 125, 134, 135). LPS elicits an increase in the generation of EC ROS (28, 117, 118, 177, 216). Interestingly, LPS-induced generation of ROS correlates with the onset of apoptosis, and agents that inhibit the formation of ROS confer protection against LPS-evoked EC apoptosis (1, 28, 117, 118, 150). Although the mechanism by which ROS contribute to LPS-induced EC apoptosis remains unknown, ROS have been shown to increase mitochondrial membrane permeability, resulting in the release of proapoptotic factors (10, 29, 63, 195, 224). One of these factors is cytochrome c, a cofactor that drives the assembly of the caspase-9-activating apoptosome (169). Furthermore, ROS have been reported to increase both the expression and activation of caspase-8 (151, 224) as well as the activation of caspase-3 (10, 224).

In addition to the generation of ROS, LPS induces the upregulation of manganese superoxide dismutase (MnSOD) (150, 206) and copper, zinc superoxide dismutase (Cu,Zn-SOD) (123). SODs serve as an important component of the EC antioxidant defense system by accelerating the conversion of superoxide to H2O2. Although both superoxide and H2O2 are potent oxidants, the latter reacts much more slowly with cellular substrates (165). LPS-induced upregulation of MnSOD is mediated, in part, by increased generation of ROS, thus SOD may act to counterbalance the proapoptotic effects of ROS (150). Consistent with this hypothesis, upregulation of SOD has been reported to inhibit apoptosis in a variety of cell types, including ECs (56, 120, 215). Furthermore, overexpression of Cu,Zn-SOD protects against reactive oxygen intermediates (ROI)-elicited induction of caspase-8, activation of caspase-3, and mitochondrial release of cytochrome c after ischemia (151, 191). Finally, overexpression of MnSOD decreases the sensitivity of EC to LPS-induced apoptosis (31). Clearly, one possible mechanism by which SOD confers protection is by restricting the accumulation of potent oxidizing molecules that may promote apoptosis. Another mechanism may be through SOD’s role in accelerating the conversion of superoxide to H2O2. Although H2O2 has been reported to induce apoptosis in some systems, others have reported that high levels of H2O2 inhibit caspase activation and apoptosis (25, 83, 131). Whether LPS-induced upregulation of SODs generates H2O2 at a level that will promote or inhibit LPS-evoked EC apoptosis remains unknown.

ROLE OF APOPTOTIC SIGNALING MOLECULES IN MEDIATING LPS-INDUCED EC ACTIVATION

A key marker of EC activation is NF-κB activation and nuclear translocation, a requisite event for many EC responses, including increased expression of cytokines and adhesion molecules. Recent studies demonstrate that several signaling molecules originally described as mediators of apoptosis also contribute to the regulation of NF-κB activation (33, 98, 103, 114, 184, 207). Evidence supporting this dual function for apoptotic signaling molecules includes: 1) enhanced caspase activation inhibits TNF-α-induced EC NF-κB nuclear translocation and VCAM-1 expression (157), 2) transient overexpression of FADD, FLIP, or caspase-8 augments basal levels of NF-κB activation (33, 98, 114), 3) overexpression of FADD upregulates monocyte chemotactic protein-1 and IL-8 expression, the transcription of which is mediated by NF-κB (184), and 4) FADD-deficient Jurkat cells display impaired activation of NF-κB after exposure to TNF-α and TNF-related apoptosis-inducing ligand (TRAIL), or Fas (207).

Similarly, evidence exists that apoptotic signaling molecules also influence the ability of LPS to induce NF-κB activation. LPS-insensitive human embryonic kidney 293T cells are sensitized to LPS-induced NF-κB activation on induced expression of Nod1, a cytoplas-
mic protein with structural homology to the apoptosis regulator, Apaf-1 (104). Furthermore, overexpression of Bcl-2 and Bcl-xL inhibits LPS-induced NF-κB activation and NF-κB-dependent gene expression in ECs (13). This inhibition of NF-κB activation corresponds with Bcl-2-mediated inhibition of IκBα degradation. The mechanism by which Bcl family members inhibit LPS-induced NF-κB activation remains unknown. Identification of a caspase consensus site within IκBα has led to speculation that caspases may be involved in its proteolysis (13). Thus Bcl-mediated inhibition of caspase activation may be expected to block IκBα degradation and subsequent activation of NF-κB.

Further evidence for cross talk between apoptotic signaling molecules and LPS-induced NF-κB activation was provided in a recent report demonstrating that the proapoptotic adapter protein FADD down-regulates LPS-induced NF-κB activation (20). In that study, overexpression of either full-length FADD or the DD of FADD in ECs blocked LPS-induced NF-κB activation. Furthermore, mouse embryo fibroblasts (MEF) derived from FADD−/− mice embryos displayed enhanced NF-κB activity relative to FADD+/+ MEF after LPS treatment. The production of IL-6 and KC, two NF-κB-dependent gene products, were similarly enhanced in FADD−/− MEF after LPS exposure relative to FADD+/+ MEF. Reconstitution of FADD in FADD−/− MEF abrogated the enhanced NF-κB activation and IL-6 and KC production elicited by LPS. Together, these data suggest that changes in FADD expression can affect LPS-induced activation of NF-κB. The role of FADD in inhibiting this event under basal conditions remains unclear as LPS activates EC NF-κB in the presence of physiological levels of FADD.

The mechanism by which FADD downregulates NF-κB-dependent gene expression remains unknown. However, the enhanced NF-κB activity in the FADD−/− MEF after LPS stimulation correlated with enhanced degradation of the inhibitor of NF-κB, IκB, suggesting that FADD exerts its effect upstream of IκB degradation. Interestingly, NF-κB activation by IL-1β, a proinflammatory cytokine that shares the same intracellular signaling pathway leading to NF-κB activation as that of LPS, is similarly downregulated by FADD (20).

The data that FADD inhibition of LPS and IL-1β-induced NF-κB activation occurs upstream of IκB degradation and that FADD inhibits a signaling pathway shared by these two distinct stimuli suggests that FADD may exert its effect through inhibiting and/or sequestering signaling molecules involved in both LPS and IL-1β-induced NF-κB activation. A proximal event in LPS and IL-1β signaling is MyD88 recruitment of IRAK through reciprocal binding of the DD regions on each of these molecules (48, 194, 212) (Fig. 4). One possibility is that FADD, another DD-containing protein, may bind and/or sequester MyD88 and/or IRAK-1 through homophilic DD-DD interactions. In fact, overexpressed MyD88 reportedly binds FADD via DD-mediated interactions (5, 92). Whether this interaction occurs when these proteins are expressed at physiological levels is not known. It has been well established that both MyD88 and IRAK are required for optimal LPS-induced NF-κB activation and NF-κB-dependent gene expression (115, 194, 222). Thus any protein that can interfere with the recruitment of these molecules to the Tlr-4 receptor-signaling complex, perhaps through DD-DD interactions, would be expected to disrupt LPS-induced NF-κB signaling. FADD contains an additional protein-binding domain, the DED (Fig. 1), which facilitates recruitment of FLIP and caspase-8 (Fig. 4). FLIP and caspase-8 have been demonstrated to bind NIK and IKK and to potentiate NF-κB signaling. The binding of the molecules is mediated, in part, by the DED regions of FLIP and caspase-8. Whether FADD can sequester NIK and IKK either through direct binding with these molecules via its own DED or indirectly through binding of FLIP and caspase-8 remains unknown.

**FUTURE DIRECTIONS**

The unique position of the endothelium at the blood-extravascular tissue interface exposes ECs to an array of circulating mediators that may be injurious. ECs...
LPS is a key proinflammatory mediator that contributes, at least in part, to the deleterious effects of gram-negative bacteremia and its attendant endotoxemia. LPS is a potent activator of the vascular endothelium and elicits an array of EC responses, including apoptosis. Key areas of future study include: 1) identifying the adapter proteins that link the Tlr-4 signaling molecules to activation of caspases (Fig. 2), 2) determining whether FLIP and Bcl-2 homologs participate in redundant cytoprotective pathways and identifying the mechanism by which these molecules confer EC protection against LPS-induced apoptosis, 3) evaluating the cross talk between apoptotic signaling molecules and NF-κB activation with the goal of devising a common therapeutic intervention that could dampen an excessive host inflammatory response and simultaneously protect against host tissue injury (Fig. 4), 4) investigating the synergistic effect that endogenous proinflammatory mediators may have with LPS and one another on promoting EC apoptosis, and 5) extending the promising though limited research evaluating the efficacy of caspase inhibitors in sepsis models.

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