Two Distinct Phospholipases C of Listeria monocytogenes Induce Ceramide Generation, Nuclear Factor-κB Activation, and E-Selectin Expression in Human Endothelial Cells

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Two Distinct Phospholipases C of Listeria monocytogenes Induce Ceramide Generation, Nuclear Factor-κB Activation, and E-Selectin Expression in Human Endothelial Cells¹

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Infection of endothelial cells by Listeria monocytogenes is an essential step in the pathogenesis of listeriosis. We recently reported that L. monocytogenes induces up-regulation of E-selectin and other endothelial adhesion molecules and subsequent polymorphonuclear leukocyte (PMN) adhesion into cultured human endothelial cells. In the present study, we characterized the mechanisms of enhanced E-selectin expression using L. monocytogenes wild type (EGD), the isogenic in-frame deletion mutants for phosphatidylycholine (PC)- and phosphatidylinositol (PI)-specific phospholipases EGDΔplcA and EGDΔplcB, as well as the nonvirulent control strain Listeria innocua. Infection of endothelial cells with EGDΔplcA or EGDΔplcB for 6 h induced, as compared with EGD wild type, intermediate levels of E-selectin mRNA and protein as well as PMN rolling and adhesion at a shear rate of 1 dyne/cm², indicating that both bacterial phospholipases are required for a maximal effect. Similarly, ceramide content and NF-κB activity were increased in L. monocytogenes-exposed endothelial cells, but only to intermediate levels for PC- or PI-phospholipase C (PLC)-deficient listerial mutants. Phospholipase effects could be mimicked by exogenously added ceramides or bacterial sphingomyelinase. The data presented indicate that PI-PLC and PC-PLC are important virulence factors for L. monocytogenes infections that induce accumulation of ceramides that in turn may act as second messengers to control host cell signal-transduction pathways leading to persistent NF-κB activation, increased E-selectin expression, and enhanced PMN rolling/adhesion. The ability of L. monocytogenes to stimulate PMN adhesion to endothelial cells may be an important mechanism in the pathogenesis of severe listeriosis. *The Journal of Immunology, 1998, 161: 3010–3018.

Listeria monocytogenes, a Gram-positive facultative intracellular bacteria, is an opportunistic pathogen for animals and humans (1). It causes food-borne septicemia and meningitis primarily in immunocompromised hosts, pregnant women, and neonates (2–4). During the course of systemic disease, many cell types are infected, including intestinal epithelial cells (5), macrophages (6), endothelial cells (7), hepatocytes (8), and fibroblasts (9), demonstrating the capability of Listeria to invade different professional and nonprofessional phagocytes. The infectious process of L. monocytogenes can be separated into the following steps: adhesion, invasion, escape from the phagosomal compartment, intracytosolic replication, and cell-to-cell spread. Recently, several genes encoding virulence determinants have been identified and found to be clustered on the chromosome. Among these are hly, plcA, and plcB, which encode the pore-forming listeriolysin (LLO), a phosphatidylinositol-specific phospholipase C (PI-PLC), and a phosphatidylcholine-specific phospholipase C (PC-PLC), respectively. These three virulence factors contribute, partly in a synergistic manner, to the lysis of the phagosome as well as to cell-to-cell spread (10–12).

Recent studies demonstrate that invasion and activation of endothelial cells by L. monocytogenes are critical events in the pathogenesis of listeriosis (13–16). In major manifestations of this disease, such as meningitis and neonatal sepsis, bacteria must cross the endothelial barrier, thereby promoting the infection of different tissues and organs. Therefore, an understanding of the interaction between bacteria and endothelial cells is relevant to the pathophysiology of listeriosis.

While antilisterial immunity is accomplished primarily by T lymphocytes (17, 18), phagocytes such as macrophages and polymorphonuclear leukocytes (PMN) also contribute to the antilisterial resistance (19–21). Adhesion of circulating leukocytes to endothelial cells is an early step in an inflammatory reaction, and we have recently reported an increased expression of different adhesion molecules (ICAM-1 and VCAM-1 as well as E- and P-selectin) on cultured human endothelial cells after infection with L. monocytogenes, an effect that was accompanied by an enhanced adhesion of PMN (14).

A prerequisite for the expression of E-selectin and other proinflammatory mediators is activation and subsequent translocation of

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1 Abbreviations used in this paper: LLO, listeriolysin; DAG, diacylglycerol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPTLC, high performance thin layer chromatography; I-κB, inhibitory-κB, PC, phosphatidylycholine; PL, phosphatidylinositol; PLC, phospholipase C; PMN, polymorphonuclear leukocyte; SMase, sphingomyelinase.
the transcription factor NF-κB from the cytoplasm to the nucleus, where it regulates the activity of different genes involved in the immune response (22–24). NF-κB is a member of the Rel family of transcriptional activator proteins (25). It is sequestered in the cytoplasm as a p50-p65 heterodimer, which is associated with two major inhibitory proteins, I-κBα and I-κBβ (26). Stimulation of cells leads to phosphorylation, polyubiquitination, and degradation of the inhibitory proteins, which results in a NF-κB activation (27–30).

*L. monocytogenes* wild-type related NF-κB translocation into the nucleus of endothelial cells was demonstrated recently by fluorescence microscopy (13). In P388D1 macrophages, *L. monocytogenes*-induced NF-κB activation was biphasic (31). The initial transient translocation of NF-κB was induced by lipoteichoic acid, which was followed by a second persistent phase mediated by expression of lissarial phospholipases and paralleled by I-κBβ degradation (31).

Ceramides are key mediators in coordinating cellular responses. They are generated via the sphingomyelinase pathway, which in turn is activated by the PLC product diacylglycerol (DAG) (32). NF-κB activation can occur in the presence of ceramides, suggesting that these sphingomyelinase products may represent the link between lissarial PLC and endothelial E-selectin expression.

In the present study, besides the wild-type *L. monocytogenes* (EGD) and the nonpathogenetic *Listeria innocua* (INN), we made use of genetically engineered mutants that lack the genes required for the expression of PI- or PC-PLC. The data presented suggest that both PLCs of *L. monocytogenes* are necessary for maximal intracellular ceramide generation, NF-κB activation, and subsequent E-selectin expression. Moreover, addition of exogenous sphingomyelinase as well as ceramides to endothelial cells mimicked the effects of *L. monocytogenes* infection with respect to E-selectin expression. We therefore suggest that lissarial PLC-induced ceramides act as potential second messengers in *L. monocytogenes*-infected endothelial cells.

**Materials and Methods**

**Materials**

Tissue culture plasticware was obtained from Becton Dickinson (Heidelberg, Germany) and Nunc (Wiesbaden, Germany). MCDB 131 medium, HBSS, PBS, trypsin–EDTA solution, HEPES, and FCS were from Life Technologies (Karlsruhe, Germany). Collagenase (CLS type II) was purchased from Worthington Biochemical (Freehold, NJ). Paraformaldehyde and Silica 60 high performance thin layer chromatography (HPTLC) plates were obtained from Merck (Darmstadt, Germany). Sodium chromate (\(\text{Cr}_2\text{O}_3\)) was purchased from New England Nuclear (Detroit, MI) at 37°C and were used in the logarithmic phase of growth.

Generation of isogenic mutant: The wild-type *L. monocytogenes* serotype 1/2a strain EGD, the *L. innocua* serotype 6b strain ATCC 33090 (INN), and the in-frame deletion mutants *L. monocytogenes* ΔplcB have been described previously (16, 36–38). To generate the isogenic mutant strain ΔplcA, the flanking regions of the plcA gene (formerly *pic* gene, accession number X54618) had been amplified separately by PCR using chromosomal DNA from *L. monocytogenes* as template. The upstream region of the *plcA* gene was amplified with the oligonucleotide pair 238 (5′-TTCCTAAAAGTGACAGGACATGC-3′) and 3974 (5′-GTTCCGAGTGAGGAGGCTAAATCATG-3′) and the downstream region of the oligonucleotide pair 3973 (5′-TGTTGTACAGGACATTCATTCATTAGGG-3′) and 1085 (5′-CATGGTTGTTCACTCTTCTTAC-3′), resulting in products of 1055 and 378 bp, respectively. Both PCR products were ligated and again amplified by PCR with the flanking oligonucleotides 238 and 1085 to obtain a DNA fragment of 1433 bp. Finally, plcA lacked the amino acids 46 to 290. The 1433-bp-long PCR product had been directly cloned into the Smal restriction site of the multiple cloning site of the vector pUC18-generating plasmid pUC18-ΔplcA. The deletion allele of the *plcA* gene, located on a 1.5-kb large Xbal/SauI restriction fragment from plasmid pUC18- ΔplcA, was cloned into the restriction sites XbaI and SauI from suizid vector paul-A (39) to obtain plasmid paulA-ΔplcA. After transformation of the wild-type strain EGD with paulA-ΔplcA, the plcA deletion mutant was subsequently generated by procedures described previously (40, 41). Verification of the ΔplcA mutant came from correlative data obtained by sequencing the chromosomal deletion generated, as well as by examining production with mAbs directed against PlcA (data not shown).

**Listeria infection assay**

Before infection with different *Listeria* strains, HUVEC were washed thrice with MCDB 131 medium without supplements or antibiotics. Bacterial concentrations from experimental cultures were adjusted by determining the OD at 600 nm, and appropriate dilutions were prepared. Then bacteria were centrifuged at 8000 rpm for 2 min, followed by two washes in PBS and one washing in plain MCDB 131 medium, and added in a bacteria to eukaryotic cell ratio of 10:1 directly to the HUVEC monolayer culture. After 2 h, the plates or flasks were washed extensively with plain medium, and subsequently cells were incubated in medium containing 50 μg/ml gentamicin to kill remaining extracellular bacteria. At times indicated in the figure legends, cells were processed for ceramide and NF-κB reporter gene assay, Northern blot for E-selectin, cell surface ELISA, neutrophil adhesion assay under stationary or flow conditions, and neutrophil rolling assay.

**Assay for ceramide content**

Ceramide content of endothelial cells preincubated with different *Listeria* strains or nonstimulated control cells was determined by HPTLC. Fluorometric quantification of ceramide was done by scanning at 254 nm using a TLC scanner (Camag). Standard ceramide curves were obtained for each ceramide standard procedure was modified by the addition of a second HPTLC run to clearly describe (43). Total ceramides were separated from other lipids on HPTLC. Ceramide content of endothelial cells preincubated with different bacterial concentrations from experimental cultures were adjusted by determining the bacteria to eukaryotic cell ratio of 10:1 directly to the HUVEC monolayer.

**Materials**

Tissue culture plasticware was obtained from Becton Dickinson (Heidelberg, Germany) and Nunc (Wiesbaden, Germany). MCDB 131 medium, HBSS, PBS, trypsin–EDTA solution, HEPES, and FCS were from Life Technologies (Karlsruhe, Germany). Collagenase (CLS type II) was purchased from Worthington Biochemical (Freehold, NJ). Paraformaldehyde and Silica 60 high performance thin layer chromatography (HPTLC) plates were obtained from Merck (Darmstadt, Germany). Sodium chromate (\(\text{Cr}_2\text{O}_3\)) was purchased from New England Nuclear (Detroit, MI). Ceramides \(\text{C}_4\) and \(\text{C}_6\) were obtained from Boimol (Hamburg, Germany). All other reagents were obtained from Sigma (Munich, Germany).

**Monoclonal Abs**

Purified freeze-dried mAb directed against E-selectin (1.2B6) was obtained from Immunotech (Marseille, France). Horseradish peroxidase-conjugated polyclonal sheep anti-mouse IgG Abs were from Amersham (Dreieich, Germany). All Abs used were azide free. To further characterize the adhesion system, endothelial cells were preincubated with 50 μg/ml inhibitory mAb for 45 min.

**Preparation of HUVEC**

Cells were isolated from umbilical cord veins and identified according to the method of Jaffe et al. (33). Briefly, cells obtained from collagenase digestion were washed, resuspended in MCDB 131/5% FCS, and seeded into well plates or flasks. Only confluent monolayers of primary cultures were used.

**Isolation and labeling of human PMN**

Heparinized human donor blood was centrifuged in a discontinuous Percoll gradient to yield a PMN fraction of >97% purity (34). Freshly isolated neutrophils were radiolabeled with \(^{51}\text{Cr}\), according to Gallin et al. (35). Briefly, after isolation PMN were incubated with 100 μCi \(^{51}\text{Cr}\) at 37°C for 1 h in RPMI 1640 medium containing 10% FCS. Subsequently, cells were washed twice in HBSS (with calcium, without magnesium) to remove unincorporated \(^{51}\text{Cr}\).

**Bacterial strains and growth media**

Bacteria were grown in brain heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C and were used in the logarithmic phase of growth.

Ceramide spots identified via corresponding ceramide standard were used to determine unequivocal ceramide generation (31). The initial transient translocation of NF-κB was induced by lipoteichoic acid, which was followed by a second persistent phase mediated by expression of lissarial phospholipases and paralleled by I-κBβ degradation (31).

Ceramide content of endothelial cells preincubated with different *Listeria* strains or nonstimulated control cells was determined by HPTLC. Fluorometric quantification of ceramide was done by scanning at 254 nm using a TLC scanner (Camag). Standard ceramide curves were obtained for...
each HPTLC run was determined by gas-chromatographic analysis of 100 μg ceramide (C₄₀) standard in relation to 20 μg 15:0 fatty acid methyl ester as internal standard, and amounted to 83 ± 7.4%.

**NF-κB reporter gene assay**

The minimal promoter vector, pGL3.BG, was created by inserting a XhoI/HindIII fragment, which contains the rabbit β-globin TATA box and transcription start (bases −43/+5), from pADneo2BGluci (47) into XhoI/HindIII-digested pGL3basic luciferase vector (Promega, Madison, WI). Subsequently, pGL3.BG was opened with SmaI, and after calf alkaline phosphatase treatment (Promega), ligated with phosphorylated double-stranded oligonucleotides containing either three copies of the consensus NF-κB recognition sequence (sense strand only underlined), 5′-GGG GAC TTT CCC TGG GGA CTT TTC TGC GGG ACT TTC CC-3′, or three copies of the mutated sequence (mutated bases underlined), 5′-GGG GCAC TTT CCC TGG GGA CTT TTC TGC GGG ACT TTC CC-3′. Recombinants containing two tandem repeats of the respective oligonucleotides were selected to create pGL3.BG.6xB and pGL3.BG.6xB-mut, and in each case identity was confirmed by double-stranded sequencing.

HUVeC were transiently transfected with 2 μg of the NF-κB plasmids pGL3.BG.6xB or pGL3.BG.6xB-mut (as negative control) using TranspoTransfection Reagent (Promega). Transfected HUVeC were stimulated for 6 h and harvested in reporter lysis buffer (Promega), and total protein was measured using the Bio-Rad reagent (Bio-Rad, München, Germany). NF-κB-luciferase assay was performed using a commercial kit (Promega). Luminescence was measured on a Lumat LB 9501 luminometer (Berthold, Bad Wildbad, Germany). Relative luminescence readings were normalized to total protein.

**Northern blot analysis**

RNA was extracted using the guanidinium isothiocyanate method, as described by Chomczynski and Sacchi (48). Total RNA was quantified by measuring absorbance at 260 nm with a Uvikon 860 spectrophotometer. RNA samples (10 μg/lane) were electroblotted onto Magna nylon membrane (MSI, Westborough, MA), and fixed by exposure to paraformaldehyde for 15 min. Human Ig was used to reduce nonspecific binding, and primary Ab was added for 30 min. Thereafter, cells were washed thrice and exposed to a horseradish peroxidase-conjugated rabbit anti-mouse Ig Ab for 30 min. After washing, o-phenylenediamine was added for 5 min. Data are indicated as OD at 492 nm.

**Cell surface ELISA for E-selectin expression**

E-selectin expression on endothelial cells preincubated with different *Listeria* strains was determined by cell surface ELISA (14, 34). Confluent, preincubated HUVeC monolayers in 96-well flat-bottom microtiter plates were washed and finally fixed with 4% paraformaldehyde for 15 min. Human Ig was used to reduce nonspecific binding, and primary Abs were added for 30 min. Thereafter, cells were washed thrice and exposed to a horseradish peroxidase-conjugated rabbit anti-mouse Ig Ab for 30 min. After washing, o-phenylenediamine was added for 5 min. Data are indicated as OD at 492 nm.

**Neutrophil adhesion assay**

After the preincubation of the HUVeC with different *Listeria* strains, medium was aspirated and endothelial cells were washed twice with MCDB 131 medium. A total of 1 × 10⁶ 51Cr-labeled PMN in 1 ml medium was added to each well (24-well plate) and allowed to settle for 30 min at 37°C and 5% CO₂. Subsequently, unbound PMN were removed by gentle aspiration, and each well was washed twice with HBSS. Adherent PMN and endothelial cells were lysed with 2 M H₂SO₄ for 30 min. Radioactivity of the lysate was quantitated with a gamma counter (Cobra Auto gamma B5003; Canberra Packard, Frankfurt, Germany). Percentage of PMN adhesion was calculated as the %Cr fraction in the lysate in relation to the total radioactivity added. Frequent counting of adhering PMN in several high power fields in the microscope confirmed that *L. monocytogenes*-related 51Cr retention in the culture wells accurately reflected neutrophil adhesion to endothelial cells.

**Neutrophil rolling and adhesion assay under flow conditions**

Leukocyte rolling and adhesion were determined using a parallel plate flow chamber, according to the method of Lawrence and Springer (50). Confluent endothelial monolayers grown on Thermanox coverslips (22 × 60 mm; Nunc, Wiesbaden, Germany) were preincubated with wild-type and different deletion mutants of *L. monocytogenes*, as described above, and subsequently treated with culture medium alone or medium containing saturating concentration of mAb against E-selectin. A suspension of 3 × 10⁴ leukocytes/ml was perfused through the chamber at a constant wall shear stress of 1 dyne/cm² (syringe pump sp100i; WPI, Sarasota, FL). Interactions were visualized using a phase-contrast videomicroscope (with a KP-7500EG; JVC Friedberg, Germany) the entire time period of leukocyte perfusion. Images were recorded at real time and played back at six- or ninefold slower speed. The tape was paused to mark the location of cells, and the displacement of the center of individual cells was measured 2 to 4 s later. Rolling was expressed as the number of rolling cells/high power field during a 3-min observation period (51). Leukocytes were considered to be adherent after 30 s of stable contact with the monolayer. Adhesion was determined after 5 min of perfusion by analysis of 10 to 12 high power (>×40) fields from videotape (50).

**Statistical methods**

Depending on the number of groups and number of different time points studied, data of Figure 3 were analyzed by a two-way ANOVA. A one-way ANOVA was used for data of Figures 1 and 2 and 4 through 7. Main effects of the studied, data of Figure 3 were analyzed by a two-way ANOVA. A one-way ANOVA was used for data of Figures 1 and 2 and 4 through 7. Main effects were then compared by an F probability test. p < 0.05 was considered significant.

**Results**

**PC-PLC and PI-PLC activity of *L. monocytogenes* were required for maximal E-selectin expression**

We recently reported an increased expression of adhesion molecules and enhanced PMN adherence in cultured human endothelial cells infected with *L. monocytogenes* (14). Maximal effects had occurred with a bacteria to cell ratio of 10:1, and this bacterial concentration was therefore used throughout the present study. The expression of internalin B, a listerial cell wall-associated protein, is mandatory for invasion and infection of endothelial cells (HUVeC) with *L. monocytogenes* (16). Since all deletion mutants used in the present study were isogenic mutants, the level of internalin B expression was equal to wild-type *Listeria*. This aspect
was also assessed by infection studies on endothelial cells (data not shown).

In HUVEC exposed, the _L. monocytogenes_ in-frame deletion mutants ΔplcA and ΔplcB as compared with the _Listeria_ wild-type EGD, an intermediate increase in E-selectin expression was demonstrated after 6 h (Fig. 1, left panel). There was no significant difference with respect to E-selectin expression between _L. monocytogenes_ ΔplcA- and ΔplcB-infected HUVEC, indicating that both phospholipases PI-PLC and PC-PLC are required for a full response (Fig. 1, left). The nonpathogenic _L. innocua_ strain (INN), also used as control, induced only a small, yet significant, up-regulation of E-selectin (INN vs non: OD 0.21 ± 0.018 vs 0.164 ± 0.007; n = 6) (Fig. 1, left panel).

The elevated E-selectin expression was accompanied by an increase of PMN adhesion in _L. monocytogenes_-exposed HUVEC 6 h postinfection, as demonstrated in the stationary adhesion assay (Fig. 1, right). Moreover, enhanced PMN rolling and adhesion was verified for infected endothelial cells under flow conditions at a shear rate of 1 dyne/cm², as determined in the parallel plate flow chamber (Fig. 2, left and right). PMN rolling (left) and adhesion (right) in ΔplcA- or ΔplcB _L. monocytogenes_-infected HUVEC was clearly higher than in endothelial cells exposed to _L. innocua_, but significantly lower as compared with EGD-stimulated monolayers. PMN rolling and PMN adherence under physiologic flow conditions were reduced in EGD-infected HUVEC in the presence of an anti-E-selectin Ab by 51% ± 4.7 and 62% ± 5.27, respectively, indicating that E-selectin was the most important endothelial adhesion molecule 6 h after _L. monocytogenes_ infection (for role of other adhesion molecules, see discussion below and also Ref. 14).

**FIGURE 1.** Left, Enhanced E-selectin expression in _L. monocytogenes_-infected HUVEC. _L. monocytogenes_ wild-type (EGD), _L. innocua_ (INN), and the deletion mutants EGDΔplcA and EGDΔplcB were incubated with HUVECs in antibiotic-free medium in 96-well plates in a bacteria to cell ratio of 10:1. After 2 h, wells were washed thrice with fresh medium supplemented with 50 μg/ml gentamicin to kill remaining extracellular bacteria. After incubation with medium containing gentamicin for another 4 h, cells were washed and processed for E-selectin cell surface ELISA. Data presented are mean ± SEM of six separate experiments. Right, Enhanced PMN adhesion to _L. monocytogenes_-infected HUVEC under stationary conditions: _Listeria_ (10:1 bacteria:cell ratio) were added directly to medium of endothelial cells in 24-well plates. After 2 h, wells were washed thrice with fresh medium supplemented with 50 μg/ml gentamicin. After another 4 h, cells were washed again and processed for neutrophil adhesion assay. A total of 1 × 10⁶ ⁵¹Cr-labeled PMN in 1 ml medium was added to each well and allowed to adhere for 30 min. Subsequently, unbound PMN were removed by gentle aspiration. Adherent PMN and endothelial cells were lysed with H₂SO₄. Percentage of PMN adhesion was calculated as the ⁵¹Cr fraction in the lysate in relation to the total radioactivity added. Data presented are mean ± SEM of three separate experiments.

**FIGURE 2.** Enhanced PMN rolling (left) and increased PMN adhesion (right) onto _Listeria_-stimulated HUVEC under flow conditions at a shear rate of 1 dyne/cm²: Different _Listeria_ strains were added to endothelial cells in four-well plates containing rectangular Thermanox coverslips (for details, see Fig. 1). After 6 h, coverslips were processed for laminar flow adhesion assay. A total of 3 × 10⁶ PMN/ml was injected into the flow system and perfused over endothelial cell monolayer for 5 min using a high precision syringe pump. Rolling PMNs (for definition and details, see Materials and Methods) were counted over a 3-min observation period (left). Adherent PMN were determined by counting 10 to 12 random high power fields (right). Data presented are mean ± SEM of five separate experiments. *Denotes experiments with endothelial cell monolayers pretreated with anti-E-selectin mAb.
**L. monocytogenes** increased intracellular ceramide level in HUVEC

Increased intracellular ceramide levels were noted in all virulent **L. monocytogenes**-infected HUVEC within 6 h (Fig. 3). **L. monocytogenes** wild-type (EGD) was most effective with respect to this ceramide increase, while **L. innocua** had a minimal effect. The phospholipase deletion mutants ΔplcA and ΔplcB induced half-maximal cellular ceramide levels without a significant difference between them. Taken together, these data suggest that both bacterial phospholipases are involved in intracellular ceramide generation.

**L. monocytogenes** infection induced persistent NF-κB activation in HUVEC

Infection of HUVEC with wild-type **L. monocytogenes** (EGD) for 6 h induced a 4.4-fold increase of the NF-κB reporter gene activity as compared with **L. innocua**-infected endothelial cells (Fig. 4). **L. monocytogenes** deletion mutants ΔplcA and ΔplcB, lacking either PI-PLC or PC-PLC, were not as effective as the wild-type **Listeria** strain (EGD), inducing an intermediate level of the NF-κB reporter gene activity, which was 2.3 (ΔplcA) and 2.1 (ΔplcB) times higher than the activity in **L. innocua**-infected cells (Fig. 4). EGD-stimulated HUVEC, which had been transiently transfected in parallel with the mutated NF-κB-luciferase plasmid (pGL3.BG.6κB-mut), revealed no detectable luciferase activity (data not shown).

**L. monocytogenes** generated ceramides as possible second messengers for increased E-selectin expression in HUVEC

Cell membrane-permeable ceramides as well as exogeneous SMase (*Staphylococcus aureus*) were added to endothelial cells to elucidate the potential role of ceramides for NF-κB activation and E-selectin expression in **L. monocytogenes**-infected cells. Incubation of endothelial cells with SMase or ceramides (C₈, C₁₆) for 4 h increased E-selectin expression in a dose-dependent manner (Fig. 5) with maximal effects seen at 1 U/ml SMase and 1 μM C₈ or C₁₆ ceramide.

These effects were accompanied by increased PMN rolling and PMN adhesion (Fig. 6, left and right), phenomena that were reduced by >70% in the presence of an anti-E-selectin mAb (Fig. 6). Exogenously applied SMase and ceramides also significantly increased NF-κB reporter gene activity (see Fig. 7). Again, SMase- or ceramide-stimulated HUVEC transiently transfected with the

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**FIGURE 3.** Increased ceramide content in **L. monocytogenes**-infected HUVEC after 2 to 6 h. Endothelial cells in two T₈₀ flasks were scraped off with ice-cold methanol. After lipid extraction, ceramide and phospholipid content were determined. Resting cells displayed 6.6 ± 0.24 ng ceramide/1 μg phospholipid. Percentage of ceramide increase in stimulated cells was calculated in relation to control cells. Data presented are mean ± SEM of five separate experiments.

**FIGURE 4.** Enhanced levels of NF-κB-reporter gene activation in **L. monocytogenes**-infected HUVEC: Different **Listeria** strains were added to endothelial cells that had been transiently transfected with a NF-κB-directed luciferase reporter plasmid (for details, see Materials and Methods). After 6 h of **Listeria** infection, cells were washed and NF-κB activity was quantitated as chemoluminescence intensity of the reporter gene-luciferase assays/μg cell protein. Data presented are mean ± SEM of five separate experiments.

**FIGURE 5.** Enhanced E-selectin expression in HUVEC stimulated with exogenously added sphingomyelinase (0.5 and 1 U/ml), C₁₆ ceramide (0.1 and 1 μM), or purified LLO (500 ng/ml). White bars indicate addition of LLO or coaddition of LLO and SMase, as well as LLO and C₁₆ ceramide. Ethanol in comparable amounts was used as control (E). After 4 h, 96-well plates were washed and processed for E-selectin cell surface ELISA. Data presented are mean ± SEM of four separate experiments.
mutated NF-κB-luciferase plasmid (pGL3.BG.6κB-mut) showed no detectable luciferase activity (data not shown).

Based on the published synergism between LLO as a pore-forming toxin and exogenously added PlcA (15) with respect to phosphoinositol metabolism, we examined whether there was also a synergistic interaction between LLO and SMase or between LLO and C_16 ceramides. As demonstrated in Figure 5, coaddition of purified LLO and SMase or C_16 ceramide resulted in a merely additive (not synergistic) effect regarding E-selectin expression.

L. monocytogenes infection resulted in up-regulation of E-selectin mRNA in HUVEC

Having demonstrated that both PI- and PC-PLC induce ceramide generation, NF-κB activation, and enhanced E-selectin expression on the endothelial cell surface, we finally characterized the effects of listerial PLC on E-selectin mRNA transcription by Northern blot hybridization using a human E-selectin cDNA probe. A maximal E-selectin mRNA content in human endothelial cells was observed after stimulation with wild-type L. monocytogenes (EGD) for 4 h (Fig. 8), whereas HUVEC exposed to L. monocytogenes ΔplcA and ΔplcB displayed intermediate E-selectin mRNA levels. Cells treated with the nonpathogenic strain L. innocua for the same incubation period did not show any detectable amount of E-selectin mRNA. These molecular data indicate, very similar to the above reported functional data, that both listerial PLC are required for maximal E-selectin mRNA expression in HUVEC.

Discussion

In a previous report, we demonstrated up-regulation of endothelial adhesion molecules and subsequent increased PMN adhesion to

![FIGURE 6. Enhanced PMN rolling (left) and increased PMN adhesion (right) onto SMase (1 U/ml) or cell membrane-permeable ceramide (1 μM)-stimulated HUVEC under flow conditions with a shear rate of 1 dyne/cm². Stimuli were added to endothelial cells for 4 h in four-well plates containing rectangular Thermanox coverslips (for details, see Fig. 2). Data presented are mean ± SEM of three separate experiments. *Denotes experiments with endothelial cell monolayers pretreated with anti-E-selectin mAb.](#)

![FIGURE 7. Enhanced levels of NF-κB-reporter gene activation in SMase or C_16 and C_9 ceramide-stimulated HUVEC: 1 U/ml SMase or 1 μM ceramide was added to endothelial cells that had been transiently transfected with a NF-κB-directed luciferase reporter plasmid (for details, see Materials and Methods). After 6 h, cells were washed and NF-κB activity was quantitated as chemoluminescence intensity of the reporter gene-luciferase assays/μg cell protein. Data presented are mean ± SEM of four separate experiments.](#)

![FIGURE 8. L. monocytogenes induced increase of E-selectin mRNA in HUVEC after 4 h: Listeria were added in a bacteria to cell ratio of 10:1. Total endothelial RNA was isolated, and E-selectin mRNA levels were determined by Northern blot hybridization utilizing an E-selectin cDNA probe. E-selectin expression was normalized to the constitutively expressed message of GAPDH. Total amount is demonstrated in relation to IL-1β-induced mRNA expression after 4 h. Data presented are mean ± SEM of three separate experiments.](#)
**L. monocytogenes**-exposed HUVEC. These effects were characterized by a biphasic kinetic with an early peak at 15 to 30 min, which was P-selectin mediated and strictly dependent on the generation of the exotoxin listeriolysin (14, 34, 52–55). To better characterize the later E-selectin-dependent phase, we analyzed the role of listerial PLC in the study presented using **L. monocytogenes** EGDΔplcA or EGDΔplcB in-frame deletion mutants. We present direct evidence for an involvement of both listerial phospholipases PI-PLC, as well as PC-PLC in the up-regulation of endothelial E-selectin. Both PLC are expressed, while **L. monocytogenes** resides inside the phagocytic vacuole and contributes to an efficient lysis of the phagosome, depending on the cell type (12, 56). Listerial PI-PLC is secreted in an active state, whereas PC-PLC is secreted as an inactive proenzyme whose activation is mediated by a listerial metalloprotease and by specific host cell factors (57). PC-PLC is a broad spectrum phospholipase that generates DAG from phosphoglycerides and ceramide from sphingomyelin (12, 58). PI-PLC is a PI-specific phospholipase that also produces DAG, which in turn can activate acidic SMase. This enzyme is located primarily in the lysosomal compartment and generates ceramides from sphingomyelin (11, 59, 60). Interestingly, each PLC induced intermediate ceramide levels, suggesting that both PC- and PI-PLC are essential for a maximal effect. This is in line with the observation that high levels of intracellular ceramide were observed only after infection with the **Listeria** wild type expressing both functional phospholipases. Indeed, Smith et al. could not detect an increase in ceramide levels in J774 cells infected with mutants lacking both phospholipases (56).

Our results suggest that ceramides generated by listerial PLCs act as second messengers in the signaling pathway that via NF-κB leads to enhanced E-selectin expression. Corroborative results were obtained using a reporter gene assay for NF-κB activity and Northern blot analysis for E-selectin. Thus, high level persistent NF-κB activity or maximal E-selectin gene transcription following infection with **L. monocytogenes** wild type was observed, which was clearly diminished in strains infected with the ΔplcA and ΔplcB phospholipase deletion mutants. There is strong evidence for ceramides as mediators of NF-κB activation (31, 61–65). The mechanisms of ceramide-induced NF-κB activation are not known in detail, but may be related to stimulation of ceramide-activated protein kinase or IκB kinase (58, 66, 67).

The potential role of ceramides as second messengers is supported by studies involving exogenously added ceramide as well as sphingomyelinase. In these experiments, ceramides induced NF-κB activation/translocation, including all subsequent reactions, indicating that this lipid mediator can substitute for endogenously generated ceramide. This confirms earlier work by Modur, who demonstrated ceramide- and SMase-related activation of human endothelial cells (68). Interestingly, in our study, maximal effects were seen in the presence of **L. monocytogenes** wild type, while intermediate levels of NF-κB activation were noted with PLC deletion mutants or membrane-permeable ceramides. These data suggest that **L. monocytogenes**-associated phospholipases may have better access to their substrate pools, thereby generating more DAG and in turn ceramide as second messenger for NF-κB activation than the extracellularly applied ceramides or sphingomyelinase. This notion is supported by the recently described synergistic interaction between LLO, a pore-forming toxin, and exogenously added PI-PLC (11, 15). In the study, present coaddition of LLO and exogenous SMase or ceramide resulted in a merely additive E-selectin expression. This observation, however, is consistent because sphingomyelin is distributed predominantly in the outer leaflet of the plasma membranes, whereas PI's are located exclusively in the inner leaflet (69, 15). Therefore, it is likely that LLO will enhance the accessibility of exogenously added PI-PLC, but not of added sphingomyelinase to its respective substrate.

The level of sphingomyelinase stimulation and ceramide accumulation in **L. monocytogenes**-infected endothelial cells is reminiscent of TNF-signaling mechanisms, thus placing sphingomyelin turnover as a central early event not only in TNF-, but also in **Listeria**-induced signal transduction (58, 59).

**L. monocytogenes** as well as ceramides and sphingomyelinase not only induce NF-κB activation in HUVEC, but also E-selectin gene expression, a process normally not detected in resting endothelium, but strongly and rapidly induced by inflammatory stimuli (70). The inducibility of the E-selectin gene requires NF-κB binding to at least three of the four positive regulatory domains in the E-selectin promoter region, a condition that is also similar for the ICAM-1 and VCAM-1 promoters (24, 71).

Up-regulation of endothelial adhesion molecules due to **L. monocytogenes** infection resulted in an increased PMN adhesion to the endothelium (14). At 6 h postinfection, this effect is dominated by E-selectin, as evidenced by experiments using anti-E-selectin mAb (Fig. 2). From previous studies, it is known that the other major endothelial adhesion molecule is ICAM-1, which contributes about 40% to PMN adhesion after 6 h and 85% after 18 h of **Listeria** infection (14). In this process, PMN adhesion is also promoted by β2 integrins, namely CD11b and most importantly CD18 (14). While a direct **Listeria**-related PMN activation may occur, an indirect PMN stimulation by **Listeria**-exposed endothelial cells is also possible because interaction of neutrophil structures with E-selectin will activate β2 integrins on the neutrophil surface, which in turn bind with high affinity to ICAM-1 on the endothelium (72).

As outlined before, up-regulation of endothelial adhesion molecules due to **L. monocytogenes** infection resulted in an increased PMN adhesion to the endothelium. Infection of endothelial cells with **L. monocytogenes** EGDΔplcA or EGDΔplcB in-frame deletion mutants resulted in intermediate E-selectin levels and showed correspondingly intermediate degrees of PMN-endothelial cell interactions. The nonproducing PLC **Listeria** stain INN did not induce PMN adhesion. To extend our previous observation on PMN adhesion under stationary conditions, we also analyzed PMN-endothelial cell interaction at a well-defined shear rate of 1 dyne/cm² using a parallel plate flow chamber. This approach not only allowed the study of PMN adhesion under more physiologic conditions, but even more importantly allowed the quantitation of PMN rolling.

In a recent study, Drevets presented data suggesting that LLO is the most important virulence factor with respect to E-selectin expression in endothelial cells and PMN adhesion, while EGDΔDplcA and EGDΔDplcB mutants induced only a minor defect (73). The reasons for these quantitatively, but not qualitatively, different results are not clear. It is likely that a combination of differences in cell passage (we have used only primary HUVEC cultures) as well as culture conditions are responsible for the discrepancies observed.

Enhanced PMN adhesion to **L. monocytogenes**-exposed endothelial cells may be seen as a protective reaction in light of several studies that documented the importance of PMN in controlling early events following bacterial entry (19, 20). Hence, in the early phase of a listerial infection, PMN substantially contribute to the nonspecific antilisterial resistance, as PMN depletion within 24 h of **Listeria** inoculation rendered mice extremely sensitive to this bacteria (21). **L. monocytogenes**-exposed endothelial cells could also support the adhesion of other circulating leukocytes such as lymphocytes or monocytes, which contribute to the specific host defense (17, 18, 72, 74, 75). Infected monocytes, however, may play a double-edged role during listerial infection because they
may act as a Trojan horse to facilitate bacterial spread to endothelial cells (7).

The interpretation of our study is limited to cultured human large vessel endothelial cells. For an exact analysis of Listeria-related alterations of endothelial function in clinical disorders, it would be desirable to also study human microvascular endothelial cells of different organs. The isolation and culture of these cells in sufficient quantities, however, is difficult, and therefore the applicability of the data presented to other important anatomical sites such as the blood brain barrier must be verified in further studies.

In conclusion, up-regulation of endothelial adhesion molecules and subsequent increased PMN adhesion to L. monocytogenes-exposed HUVEC occur in a biphasic manner with an early peak at 15 to 30 min, which was P-selectin mediated and strictly dependent on the generation of lipoxygenin (14). For the second phase, which phenotypically results in E-selectin up-regulation, we now present evidence for a requirement of both (PC-PLC, PI-PLC) listerial phospholipases. A role for ceramide as a second messenger in the expression of E-selectin as an inflammatory gene product via NF-κB activation was inferred from the ability of listerial phospholipases to generate ceramide in endothelial cells. PI- and PC-phospholipase effects could be mimicked by exogenous addition of ceramides or sphingomyelinase. Therefore, we suggest ceramides could be mimicked by exogenous addition of ceramides or sphingomyelinase. Therefore, we suggest ceramides to be a missing link between Listeria infection and cellular inflammatory responses.

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LISTERIAL PHOSPHOLIPASES AND ENDOTHELIAL CELL ACTIVATION


