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Listeriolysin Is a Potent Inducer of the Phosphatidylinositol Response and Lipid Mediator Generation in Human Endothelial Cells

ULF SIBELIUS,[1] FRANK ROSE,[1] TRINAD CHAKRABORTY,[2] AYUB DARJI,[3] JÜRGEN WEHLAND,[3] SIEGFRIED WEISS,[3] WERNER SEEGER,[1] AND FRIEDRICH GRIMMINGER[1]*

Department of Internal Medicine[1] and Institute of Medical Microbiology,[2] Justus Liebig University, 35385 Giessen, and Division of Cell Biology and Immunology, National Center for Biotechnology, 38124 Braunschweig,[3] Federal Republic of Germany

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The impact of *Listeria monocytogenes* listeriolysin O (LLO) secretion on phosphoinositide metabolism and mediator (platelet-activating factor and prostaglandin I_2) generation was investigated in human umbilical vein endothelial cells. Wild-type *L. monocytogenes*, purified LLO, and an *L. innocua* strain engineered to secrete LLO all elicited a strong response, whereas mutant strains defective in LLO production were ineffective. Thus, human umbilical vein endothelial cell stimulation by listeriae is linked to production of LLO.

Listeria monocytogenes is a well-recognized human pathogen, which may cause severe illness, including sepsis, mainly in pregnant women and newborns, as well as in elderly and immunocompromised persons (5). The cell biology of *L. monocytogenes* infection can be divided into four broad stages: internalization, escape from a vacuole, nucleation of actin filaments, and cell-to-cell spread. The best-characterized determinant of *L. monocytogenes* pathogenicity is listeriolysin O (LLO), a member of the family of sulfhydryl-activated pore-forming cytolysins (11). In the present study, we investigated the role of *L. monocytogenes* LLO secretion in the induction of signalling events and inflammatory and vasoactive mediator generation, such as those encountered in sepsis, in human umbilical vein endothelial cells (HUVEC) (12).

HUVEC were cultured as previously described (8). Recombinant strains of *L. monocytogenes* and *L. innocua* were grown as previously reported (3, 4, 9); the apathogen *L. innocua* was used as the host for selective expression of the LLO gene (*hly*) and the *prfA* regulator gene (Table 1). The hemolysin assay was performed as previously described, at a final erythrocyte concentration of 0.5% (3). LLO was purified as described in reference 4. Platelet-activating factor (PAF) production in HUVEC (total cellular and extracellular PAF) was quantified by lipid extraction, SP–high-pressure liquid chromatography separation, and induction of platelet serotonin release (15), as well as by bioincorporation of radiolabeled acetate (15). Phosphoinositide (PtdIns) turnover was investigated by measuring the accumulation of inositol phosphates as described by Berridge et al. (2); the cellular phospholipid pool was prelabeled with [*myo*-^{3}H]inositol. Prostaglandin I_2 (PGI_2) was quantified by radioimmunoassay (13). Endothelium-bacterium coincubation was performed with a bacterial density of approximately 3.5×10^6/ml in the final assay volume. After various time periods, reactions were stopped by admixing trichloroacetic acid (inositol phosphates) or chloroform-methanol (PAF) or by cooling to 4°C (PGI_2).

Coincubation of HUVEC with wild-type *L. monocytogenes* (EGD+) induced an outstandingly strong PtdIns hydrolysis response (Fig. 1). The response elicited surpassed that evoked by thrombin, the most potent activator of the PtdIns hydrolysis-related signal transduction pathway in HUVEC hitherto known (7), by more than twofold and that induced by the calcium ionophore A23187 manifold. Several lines of evidence strongly suggest that expression of LLO by the listerial strain was mainly responsible for provocation of the endothelial signalling process. First, both the apathogenic strain *L. innocua* INN− (4), which is nonhemolytic and noninvasive, and a mutant strain lacking the *prfA* regulator gene (EGD*prf*A1; 9), which regulates all known virulence factors of *L. monocytogenes*, were devoid of any ability to activate the PtdIns hydrolysis pathway in HUVEC. Second, the responsiveness was virtually lost upon use of an isogenic strain, *L. monocytogenes* EGD−, which produces a truncated, nonhemolytic 40-kDa polypeptide (11). Third, when avirulent strain *L. innocua* INN+ was used as a host to express listeriolysin, the ability to induce PtdIns breakdown in HUVEC was restored. Fourth, PtdIns hydrolysis was reproduced by purified LLO in the absence of bacteria, with maximum response noted at a toxin concentration of 500 ng/ml (Fig. 2). The presence of LLO is thus evidently both a conditio sine qua non and a sufficient prerequisite for potent triggering of this important signal transduction pathway in HUVEC by listeriae.

Neither adhesion nor invasion of *L. monocytogenes* was required for induction of the PtdIns response. Microscopic examination of the endothelium-bacterium cocultures did not reveal any listerial uptake by the HUVEC within a 60-min incubation period for all of the strains investigated. Moreover, the listeriolysin-producing recombinant *L. innocua* INN+ clearly possessed the ability to induce PtdIns hydrolysis, although this mutant is unable to invade epithelial and endothelial cells. This reasoning is further supported by the efficacy of purified LLO observed in the absence of any bacteria.

The LLO-related induction of HUVEC signalling occurred in the absence of HUVEC lysis, as indicated by a low percentage of lactate dehydrogenase (LDH) release. Under the given experimental conditions, control HUVEC liberated 15% ± 4% of the total LDH content in the absence of bacteria, and the same percentage (15% ± 3%) was noted for coincubation with strain EGD+ (10-min incubation periods; $n = 4$ each; mean ± the standard error of the mean [SEM]). Indeed, the

* Corresponding author. Mailing address: Department of Internal Medicine, Klinikstraße 36, D-35392 Giessen, Federal Republic of Germany. Phone: (49) 641-702-4064. Fax: (49) 641-702-3697.

TABLE 1. *Listeria* strains used in this study[a]

Strain	Genotype	Relevant hemolytic phenotype[b]	Designation
L. monocytogenes EGD serotype 1/2a			
EGD	Wild type	+	EGD+
EGD*hly-1*	*hly-1*	−	EGD−
EGD*prfA1*	*prfA1*	−	
L. innocua NCTC 11288 serotype 6a			
11288(pERL3)	Wild type	−	INN−
11288(pERL3-501)	*prfA hly*	−	INN+

[a] Recombinant strains of *L. monocytogenes* included the EGD *hly-1* strain, which produces a truncated, nonhemolytic 40-kDa polypeptide (11), and the regulator gene mutant EGD*prfA1*. *L. innocua* was engineered to solely produce LLO (4).

[b] Hemolytic phenotypes observed on human blood agar plates were scored as follows: ++, strongly hemolytic; +, weakly hemolytic; −, nonhemolytic.

experiments with purified LLO indicated a clear window of threshold levels between the optimum exotoxin dose for induction of inositol phosphate accumulation (500 ng/ml; LDH release, <20%) and the concentration provoking cell injury (1,000 ng/ml; LDH release, >30%). Correspondingly, the *L. innocua* mutant superexpressing LLO (INN+) provoked an LDH liberation of 44% ± 6% (n = 4).

Interestingly, LDH release, but not induction of PtdIns hydrolysis, was blocked by preincubation of the hemolysin with cholesterol. Incubation of HUVEC with INN+ in the presence of 100 μg of cholesterol per ml completely suppressed LDH liberation in response to this bacterial agent to baseline values but did not block the induction of PtdIns hydrolysis (4,881 ± 335 cpm after 10 min and 5,335 ± 230 cpm after 20 min; n = 4 each; mean ± SEM). Similarly, inositol phosphate accumulation in response to 500 ng of LLO per ml was not reduced in the presence of 100 μg of cholesterol per ml. It has previously been reported that cholesterol inhibits LLO-induced cell damage in murine macrophages but not the toxin-evoked production of interleukin-1 in this cell type (17). Thus, there is growing evidence that the membrane-damaging ability of LLO can be dissociated from its capacity to induce signal transduction events, suggesting that these properties are associated with physically distinct regions within this molecule.

In accordance with the PtdIns hydrolysis, the generation of large quantities of PAF was provoked by the LLO-secreting

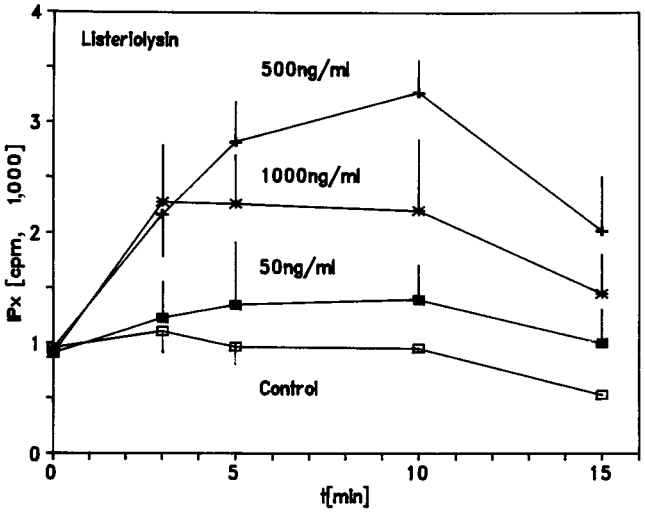

FIG. 2. Time and dose dependency of inositol phosphate (IP) accumulation in response to purified LLO. HUVEC were prelabeled with ^{3}H-inositol for 24 h and exposed to various exotoxin concentrations for different time periods. Extracted inositol phosphates (IP_3, IP_2, and IP_1) are collectively depicted as IP_x. Experiments with 500 and 1,000 ng of LLO per ml significantly differed from the control experiment.

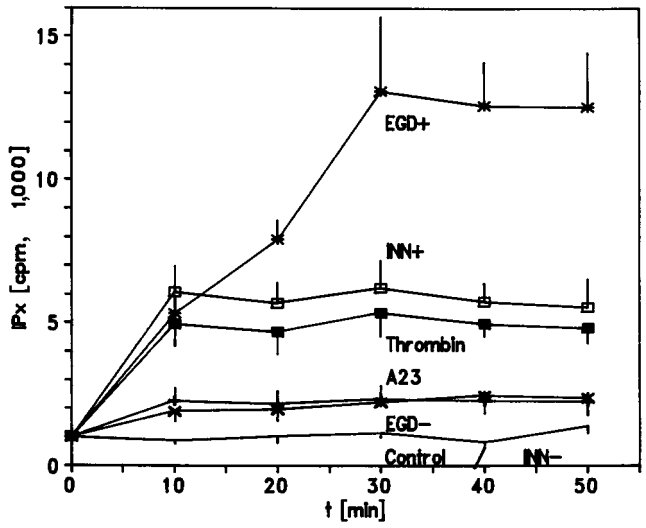

FIG. 1. Time course of inositol phosphate (IP) generation in response to various bacterial strains. HUVEC were prelabeled with ^{3}H-inositol and subsequently incubated with strain EGD+, EGD−, INN+, or INN−. All bacteria were used at 3×10^6/ml. For comparison, incubation with thrombin (0.2 U/ml) or A23187 (5 μM) or sham incubation (Control) was performed in parallel experiments. After various time periods, inositol phosphates were extracted and separated by anion-exchange chromatography. IP_3, IP_2, and IP_1 are collectively depicted as IP_x. Means ± SEM of four independent experiments each are given. Experiments with EGD+, INN+, and thrombin significantly differ from the control experiment.

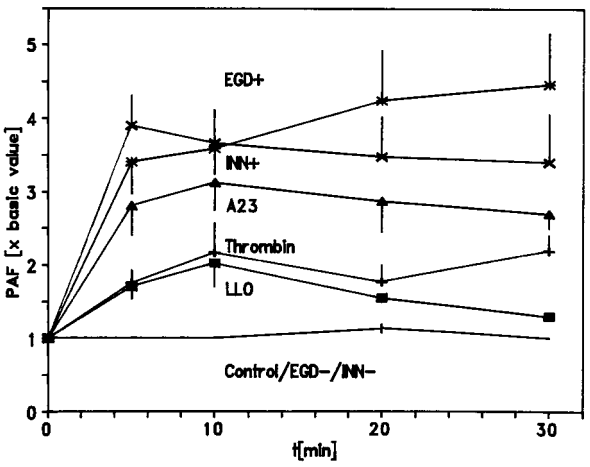

FIG. 3. Time course of PAF generation in response to various bacterial strains. HUVEC were incubated with strain EGD+, EGD−, INN+, or INN−. All bacteria were used at 3×10^6/ml. In addition, incubation with purified LLO (500 ng/ml), thrombin (1 U/ml), or A23187 (10 μM) or sham incubation (Control) was performed. After various time periods, secreted and cell-bound PAF was lipid extracted, purified by high-pressure liquid chromatography, and quantified by induction of ^{3}H-serotonin release from prelabeled platelets. Means ± SEM of four independent experiments are given. Experiments with EGD+, INN+, A23187, LLO, and thrombin significantly differed from the control experiment.

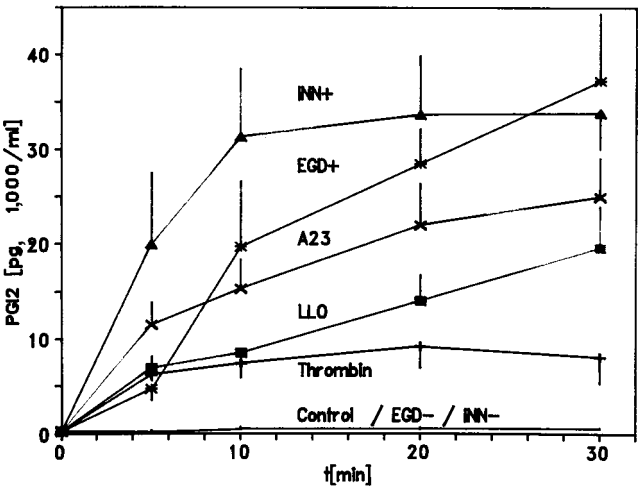

FIG. 4. Time course of PGI_2 formation in response to various bacterial strains. HUVEC were incubated with strain EGD+, EGD−, INN+, or INN−. All bacteria were used at 3×10^6/ml. In addition, incubation with purified LLO (500 ng/ml), thrombin (1 U/ml), or A23187 (10 μM) or sham incubation (Control) was performed. Prostanoids released into the supernatant were quantified after various time periods by enzyme-linked immunosorbent assay. Means ± SEM of four independent experiments are given. Experiments with EGD+, INN+, A23187, LLO, and thrombin significantly differed from the control experiment.

strains (Fig. 3). EGD+ and INN+ were most potent, followed by the calcium ionophore A23187, thrombin, and purified listeriolysin, respectively (Fig. 3). The LLO-defective strains EGD− and INN− did not provoke any PAF generation. A similar rank order of efficacy was also noted for induction of PGI_2 synthesis (Fig. 4). It may thus be assumed that the generation of inflammatory and vasoactive mediators in LLO-exposed HUVEC proceeds via stimulation of the PtdIns hydrolysis-related signal transmission sequence. Both PAF and PGI_2 are known to be synthesized in response to ligands linked to PtdIns metabolism via receptor occupancy, such as bradykinin (16), histamine, and thrombin (7). Inositol phosphate-induced Ca^{2+} liberation from nonmitochondrial Ca^{2+} stores is generally assumed to represent the link to subsequent activation of phospholipases anteceding the generation of prostanoids and PAF (1). More recently, listeriolysin-expressing bacteria have been shown to induce phosphorylation of mitogen-activated protein kinase (14).

However, we note that while listeriolysin itself was a potent inducer of PtdIns hydrolysis and the kinetics of stimulation were superimposable when evoked by either the *L. monocytogenes* wild-type strain or the *L. innocua* listeriolysin-producing recombinant, the wild-type listeriae turned out to be the more potent stimulus in the further course of HUVEC stimulation, despite stronger expression of LLO by the recombinant *L. innocua* strain. Since neither adhesion nor invasion is required to elicit this response, a further secreted soluble factor may act synergistically with listeriolysin to potentiate PtdIns hydrolysis. Candidates for such higher efficacy of the *L. monocytogenes* wild-type strain are the two distinct phospholipases C expressed by wild-type listeriae but not by the *L. innocua* listeriolysin-producing recombinant (4). Compatible with such a suggestion is the observation that the wild-type EGD strain was a more potent inducer of PtdIns hydrolysis than were optimum quantities of purified LLO.

In conclusion, the listerial virulence factor LLO, presented to the surface of HUVEC, turned out to be a potent inducer of intracellular signalling and mediator generation in this cell type. Interestingly, similar potency was recently demonstrated for the hemolysin of *Escherichia coli* in human neutrophils, resulting in degranulation, respiratory burst, and lipid mediator generation (6). Cell surface receptors for LLO have hitherto not been characterized, and further experimental approaches are necessary to delineate the molecular mechanisms through which this bacterial agent triggers the preformed signal transduction pathway in endothelial cells when offered to their outer membrane. These findings may contribute to the understanding of vasoregulatory disturbances and widespread inflammatory events encountered under conditions of severe listerial infection and sepsis.

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