Endotoxin "Priming" Potentiates Lung Vascular Abnormalities in Response to *Escherichia coli* Hemolysin: An Example of Synergism between Endo- and Exotoxin

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Summary

The pore-forming hemolysin of *Escherichia coli* (HlyA), an important virulence factor in extraintestinal *E. coli* infections, causes thromboxane generation and related vasoconstriction in perfused rabbit lungs (Seeger, W., H. Walter, N. Suttorp, M. Muhly, and S. Bhakdi. 1989. *J. Clin. Invest.* 84:220). We investigated the influence of pulmonary vascular "priming" with endotoxin on the responsiveness of the lung to a low-dose HlyA challenge. Rabbit lungs were perfused with Krebs Henseleit buffer containing 0.1–100 ng/ml *Salmonella abortus equii* lipopolysaccharide (LPS) for 60–180 min. This treatment caused protracted release of tumor necrosis factor into the recirculating medium, but did not induce significant alterations of pulmonary hemodynamics and fluid balance. At a dose of 1 ng/ml, HlyA elicited only moderate thromboxane release (<200 pg/ml) and pulmonary artery pressure increase (≤6 mmHg) in control lungs. Acceleration and potentiation of both the metabolic and vasoconstrictor response occurred in lungs primed with LPS. This priming effect displayed dose (threshold ~0.1–1 ng/ml LPS) and time dependencies (threshold ~60–90 min LPS incubation). Maximum thromboxane release and pulmonary artery pressure increase surpassed the responses to HlyA in nonprimed lungs by more than 15-fold. Cyclooxygenase inhibition and thromboxane-receptor antagonism blocked these effects. These data demonstrate that LPS priming synergizes with HlyA challenge to provoke vascular abnormalities that are possibly relevant to the pathogenesis of organ failure in severe local and systemic infections.

Severe bacterial infection and sepsis are inevitably accompanied by the liberation of toxic microbial substances, of which LPS (endotoxin) is the most widely known (1). LPS may be released from a bacterial focus or circulating bacilli, or may be resorbed in enhanced quantities from the gastrointestinal tract under conditions of compromised mucosal barrier function (2, 3). Adverse effects evoked by LPS include micro-circulatory disturbances that lead to organ failure. These events appear to result from the activation of inflammatory cells and the production of secondary endogenous mediators rather than from direct cellular toxicity of LPS (1, 4, 5). In addition, low concentrations of LPS have been found to "prime" inflammatory cells in vitro for an enhanced responsiveness to a second inflammatory stimulus, e.g., lipid and peptide chemoattractants (6–15). In perfused lungs, enhanced reactivity to platelet activating factor was observed after pretreatment of the isolated organ with endotoxin (16).

In the present study, we investigated whether LPS priming might synergize with the action of a proteinaceous, pore-forming exotoxin to cause vascular abnormalities. We employed *Escherichia coli* hemolysin (HlyA)[1] as a model toxin, since the pathogenetic relevance of this agent has been well established in animal models (17–19). An analogous role as virulence factor in human infections has been inferred from the high association of HlyA production with disease, including pyelonephritis and septicemia (18, 20, 21). HlyA exerts a particularly potent action on neutrophils, monocytes, and endothelial cells, where secondary cellular events such as generation of inflammatory mediators may be mediated not only by passive calcium flux through toxin pores, but also via trig-

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[1] *Abbreviation used in this paper:* HlyA, hemolysin.

gering of the preformed phosphatidylinositol hydrolysis–related signal transduction pathway (22–27). In human neutrophils, the toxin thus evokes degranulation, production of reactive oxygen species, and lipid mediator synthesis (23–25, 28). Interest in HlyA has been boosted by the recognition that this toxin represents the prototype of a large family of structurally and functionally related pore-forming toxins produced by other gram-negative bacterias (29–34).

To address possible cooperative effects of LPS priming and HlyA efficacy, we have employed the model of the buffer-perfused rabbit lung. This provides a means to investigate the pathogenesis of inflammatory organ injury in the absence of plasma proteins and circulating blood cells. Infusion of viable HlyA-producing *E. coli*, but not of genetically related toxin-negative strains, was previously found to provoke pulmonary hypertension and pulmonary edema in the perfused lungs (35). Similar effects were discerned when low doses of purified HlyA were applied (36). Here, we demonstrate a remarkable synergism between LPS and HlyA. Preexposure of the perfused rabbit lungs to the LPS primed the organs for markedly accelerated and enhanced responsiveness to low-dose HlyA challenge. Such synergism between endotoxin and a membrane-damaging exotoxin may be widely operative and relevant to the pathogenesis of vascular abnormalities in severe infection and sepsis.

Materials and Methods

Reagents. Goat anti-human TNF-α with established cross-reactivity with rabbit TNF-α was graciously provided by J. C. Mathison (Scripps Research Institute, La Jolla, CA), and *Salmonella abortus equii* endotoxin by C. Galanos (Max Planck Institute of Immunology, Freiburg, Germany). D,L-Lysin-mono-acetylsalicylate/glycine (9:1; ASA) was obtained from Bayer AG (Leverkusen, Germany), BM 13.505 from Boehringer AG (Mannheim, Germany), and MTT from Sigma (Munich, Germany). Rabbit anti-TxB₂ was from Paesel AG (Frankfurt, Germany), and recombinant murine TNF-α from Genzyme (Frankfurt, Germany). Tritium-labeled TxB₂ was purchased from New England Nuclear (Dreieich, Germany). A photometric test for the detection of LPS was obtained from Kabi Vitrum (Coatest endotoxin; Munich, Germany). All other biochemicals were obtained from Merck AG (Darmstadt, Germany).

Preparation of HlyA. HlyA was purified as previously described (37). The toxin was recovered in active and monomeric form, and the LPS contamination of the preparation used was $\leqslant$3 ng/μg protein.

Perfused Rabbit Lungs. The model has been previously described in detail (38–40). The lungs were perfused with Krebs Henseleit buffer through cannulas in the pulmonary artery and the left atrium (flow 100 ml/min; left atrial pressure 2 mm Hg), being placed in a temperature-equilibrated housing chamber at 37°C and freely suspended from a force transducer. They were ventilated with room air supplemented with 4% CO_2 (tidal volume 8–14 ml/kg, frequency 25–30 breaths/min; positive endexpiratory pressure of 1.5 mmHg). The alternate use of two separate perfusion circuits, each containing 250 ml, allowed exchange of perfusion fluid. Perfusion pressure, ventilation pressure, and the weight of the isolated organ were registered continuously. Lungs selected for the study were those that (*a*) had a homogenous white appearance without signs of hemostasis or edema formation; (*b*) had pulmonary artery and

ventilation pressures in the normal range; and (*c*) were isogravimetric (lung weight gain <0.3 g/h) during an initial steady state period of at least 45 min.

Biochemical Measurements. TxB₂ was assayed by RIA as described (19). TNF-α was determined in a cytolytic cell assay in the mouse fibrosarcoma cell line WEHI 164 clone 13 (kindly donated by Dr. T. Espevik, University of Trondheim, Trondheim, Norway), as previously described (41). The WEHI cells (2 × 10⁴) were incubated with serial dilutions of perfusate in microtiter wells (Nunc, Rothkilde, Denmark). After 18 h, MTT (5 mg/ml in PBS; 100 μl/well) was added. The reaction was stopped after 4 h by addition of 5% formic acid in 2-propanol, and the content of reduced MTT was read in a microELISA autoreader (570 nm). The titer of TNF-α is expressed in U/ml and was chosen as the reciprocal of the dilution necessary to cause 50% cytotoxicity. Recombinant murine TNF-α served as standard in all assays. The sensitivity of WEHI 164.13 ranged between 0.8 and 0.02 pg protein/cytolytic unit in the different experiments. Rabbit TNF-α units were expressed as picograms of murine recombinant TNF-α protein to compensate for the variance in sensitivity. To establish the nature of the cytolytic activity as TNF-α, an antiserum directed against human TNF-α with established cross-reactivity with rabbit TNF-α (42) was added to the perfusates. It was found that it neutralized all cytolytic activity measured with the WEHI cells. Potassium was measured according to standard techniques.

Experimental Protocol. After a steady-state period of 40 min, the recirculating perfusate was exchanged with fresh buffer medium, and time was set at zero. Different quantities of LPS in 500 μl PBS or the vehicle only were admixed to the buffer fluid and recirculated for 60–180 min. Thereafter, HlyA, dissolved in 250 μl saline, was bolus injected into the pulmonary artery to achieve a final buffer concentration of 1 ng/ml, and recirculation was continued for another 20 min. 1 ml-perfusate samples for determination of TxB₂ and potassium and 3-ml samples for TNF measurements were taken every hour during the period of LPS or sham priming and 2, 5, 10, and 20 min (TxB₂, K⁺) as well as 20 min (TNF) after application of HlyA.

Results

Pressor Responses. Under baseline conditions, the pulmonary artery pressure ranged between 5 and 10 mmHg in all experiments. Admixture of 0.1 and 1 ng/ml LPS to the buffer fluid did not exert any effect on the baseline vascular pressure (Fig. 1; Table 1). 10 and 100 ng/ml LPS provoked only minor increases in pressure values that never exceeded 4 mmHg.

In nonprimed lungs, bolus injection of 1 ng/ml HlyA caused a slight, protracted rise in pulmonary artery pressure, with maximum increases between 5 and 6 mmHg within 20 min. These pressor responses were potentiated in a time- and dose-dependent manner by priming with LPS. Taking 120 min as standard priming time, pressure increments up to >70 mmHg were provoked upon subsequent HlyA challenge (Figs. 1 and 2), and the vasoconstrictor response occurred much more rapidly: more than two thirds of the maximum pressure rise was complete within 1 min in lungs primed with 1 and 10 ng/ml LPS, whereas the maximum pressure elevation evoked by HlyA application in nonprimed lungs occurred after approximately 20 min.

Fig. 3 shows the time dependency of the LPS priming process, using 10 ng/ml LPS. No augmentation of the vasocon-
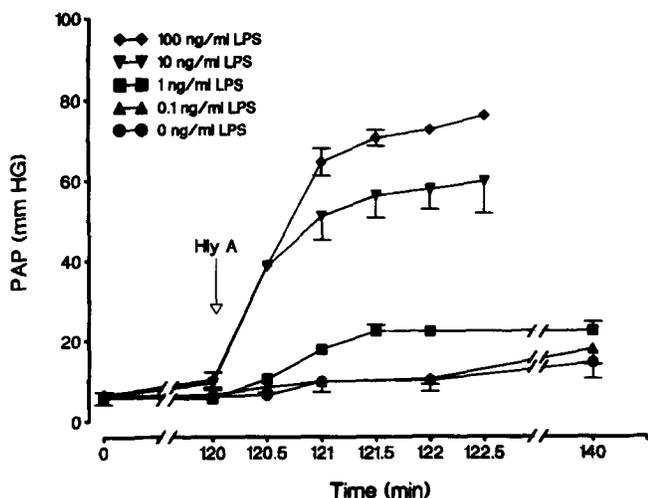
Figure 1. Pulmonary artery pressure (*PAP*) responses to 1 ng/ml HlyA in dependency on preceding LPS priming. Lungs were perfused with various LPS concentrations (given in ng/ml) for 2 h, followed by pulmonary artery injection of HlyA to achieve the intended final toxin concentration. Mean ± SEM of four independent experiments each are given (SEM bars are missing when falling into symbol). (The 122- and 122.5-min data in the group with 100 ng/ml LPS represent only two remaining lung experiments).
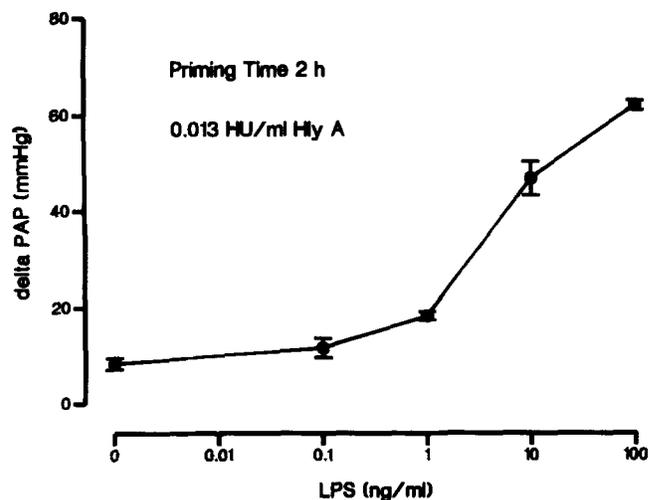

Figure 2. Dependency of the vasoconstrictor response evoked by HlyA on the LPS dose used for priming. Lungs were perfused with various LPS concentrations for 2 h, followed by pulmonary artery injection of HlyA (1 ng/ml). Maximum rises in pulmonary artery pressure (*delta PAP*) measured within 20 min after exotoxin application are given in mean ± SEM (*n* = 4 experiments each).

strictor response was noted upon simultaneous application of both LPS and HlyA. A minimal priming period of 60 min was required to provoke a moderate amplification of the pressor response, and a steep increase in priming efficacy occurred in the 60–120 min post-LPS period. Maximum responses, although showing off-leveling of the dose-dependency curve, were observed in lungs preexposed to LPS for 180 min.

Thromboxane Generation. Only minor liberation of TxB₂ was noted in control lungs and in lungs undergoing the LPS priming procedure up to 3 h (Table 2). Application of 1 ng/ml HlyA alone provoked only moderate thromboxane release

(<200 pg/ml). In LPS-primed organs, however, marked liberation of the prostanoid occurred in response to subsequent challenge with the exotoxin, whereby similar strict dependency on the priming time was noted. More than 30-fold increases in circulating TxB₂ levels were provoked within 20 min to HlyA challenge in lungs preincubated with 10 ng/ml LPS for 2 h. After 3-h priming, a greater than 10-fold increase in exotoxin-elicited TxB₂ levels occurred within only 2 min.

TNF Liberation. Lungs perfused with the buffer alone released only very small amounts of TNF into the recirculating fluid. Infusion of LPS resulted in a progressive release of this

Table 1. *Pulmonary Artery Pressure (PAP) and Lung Weight Gain (W) in LPS-exposed Lungs*

| Priming time | LPS-dose | PAP (mmHg) | | ▲ W (g) | |
		Pre-LPS	Post-LPS	Pre-LPS	Post-LPS
min	*ng/ml*				
120	0	5.5 ± 0.4	6.0 ± 0	0.03 ± 0.14	0.16 ± 0.06
120	0.1	6.3 ± 0.34	6.3 ± 0.6	−0.06 ± 0.03	1.0 ± 0.06
120	1	6.0 ± 0.57	6.3 ± 0.6	0.13 ± 0.03	0 ± 0.11
120	10	5.3 ± 0.88	8.3 ± 1.33	0.13 ± 0.09	0 ± 0.1
120	100	6.6 ± 0.33	10.0 ± 0.57	0.1 ± 0.06	0.06 ± 0.12
0	10	6.0 ± 0.58	5.6 ± 0.33	0.16 ± 0.14	−0.03 ± 0.06
60	10	6.6 ± 0.33	7.0 ± 0.58	0.06 ± 0.08	0.3 ± 0.11
90	10	6.0 ± 0.58	6.6 ± 0.89	0.16 ± 0.08	0.2 ± 0.43
120	10	5.3 ± 0.88	8.3 ± 1.33	0.13 ± 0.08	0 ± 0.1
180	10	5.0 ± 0.58	9.3 ± 0.88	0.06 ± 0.12	1.53 ± 0.96
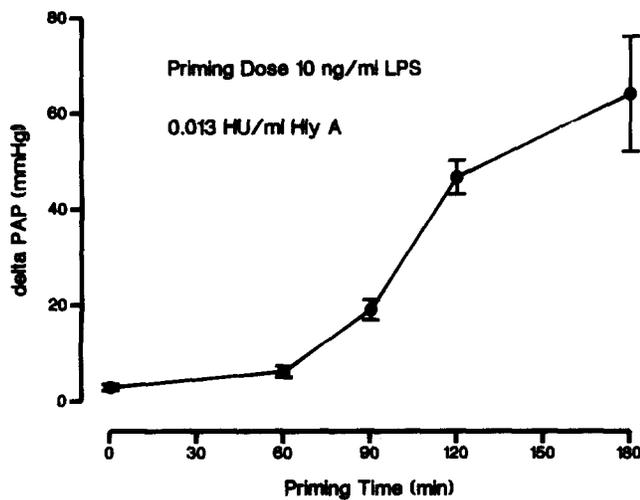
Figure 3. Time dependency of LPS priming with respect to vasoconstrictor responses provoked by HlyA. Lungs were perfused with 10 ng/ml LPS for various time periods, followed by injection of HlyA (final concentration, 1 ng/ml) into pulmonary artery. Maximum rises in pulmonary artery pressure (*delta PAP*) measured within 20 min after exotoxin application are given in mean ± SEM (n = 4 experiments each).

cytokine (time and dose dependencies are depicted in Fig. 4). A subsequent 20-min challenge with HlyA had no significant effect on the release of TNF in either primed or non-LPS–primed lungs (data not shown).

Effect of Thromboxane Inhibition on Pressor Responses. Lungs were primed with 10 ng/ml LPS for 180 min. Acetylsalicylic acid (250 μM) or BM 13.505 (10 μM; 43) was then admixed to the buffer fluid 5 min before HlyA challenge (1 ng/ml; n =4 experiments each). Rises in pulmonary artery pressure were then found to be restricted to 8.5 ± 2.5 and 6.3 ± 1.8 mmHg, respectively, as compared with 64 ± 12 mmHg in the absence of either inhibitor.

Lung Edema Formation and Potassium Liberation. Total weight gain of the isolated organs ranged below 2 g over the entire LPS priming procedure at all endotoxin doses used (Table 1). The dramatic vasoconstrictor responses elicited by HlyA in LPS-primed organs resulted in rapid edema formation at pulmonary artery pressure levels >30 mmHg. In lungs with maximum LPS priming (3-h preincubation with 10 and 100 ng/ml LPS), the exotoxin-elicited weight gain exceeded 15 g, and experiments were terminated at this level of edema formation.

LPS priming did not result in any significant liberation of potassium into the recirculating buffer medium. Intravascular application of 1 ng/ml HlyA provoked significant potas-

Table 2. *Thromboxane B_2 Generation in Response to LPS Priming and HlyA Stimulation*

			TxB$_2$-levels (pg/ml)					
						Post-HlyA		
Priming Time	LPS-dose	HlyA-dose	Pre-priming	Post-priming	2'	5	10'	20'
min	*ng/ml*	*ng/ml*						
180	0	0	87.0	99.5	103.0	100.3	91.3	79.0
			± 16	± 11	± 11	± 15	± 6	± 17
180	10	0	73.3	116.5	—	—	—	122.3
			± 15	± 18	—	—	—	± 16
180	0	1	72.2	70.2	99.4	123.8	198.0	181.2
			± 7	± 7	± 10	± 15	± 67	± 55
0	10	1	68.0	69.4	69.9	96.0	60.0	70.5
			± 21	± 12	± 15	± 11	± 16	± 19
60	10	1	66.0	64.5	81.0	105.7	120.7	162.7
			± 12	± 8	± 16	± 22	± 28	± 13
90	10	1	62.0	75.0	136.0	184.0	246.0	823.0
			± 12	± 16	± 27	± 52	± 84	± 351
120	10	1	58.0	78.0	572.0	605.0	985.0	3133.0
			± 11	± 14	± 227	± 102	± 127	± 903
180	10	1	74.0	101.0	1191.0	*	*	*
			± 8	± 17	± 304	*	*	*

* In lungs with high dose LPS priming for 3 h, HlyA stimulation provoked marked edema formation. These experiments were terminated in advance, when total lung weight gain surpassed 15 g.
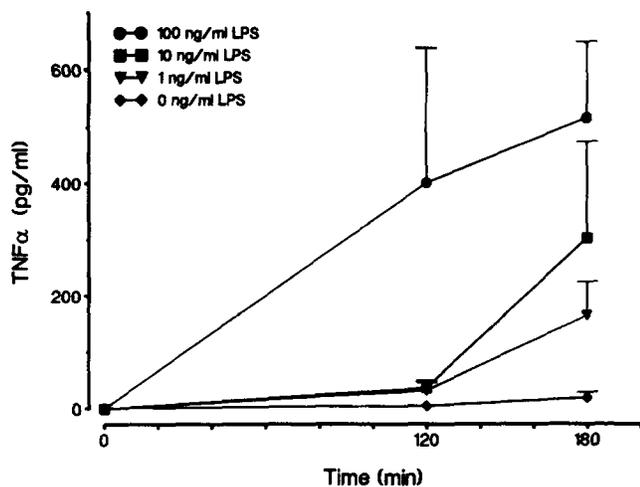
Figure 4. Liberation of TNF into the lung perfusate during LPS priming. Lungs were perfused with various LPS concentrations for 3 h. TNF concentrations in the recirculating buffer fluid are given (mean ± SEM; n = 4 experiments each).

sium release within 20 min (0.32 ± 0.04 mmol). These values were independent of LPS priming (data not shown).

Discussion

Parenteral application of LPS reproduces pathophysiologic sequelae of sepsis and ARDS in many animal models (44). The endotoxin responsiveness of the pulmonary vasculature in terms of vasoconstriction and vascular leakage is, however, species dependent. In the present investigation, no or only very minor changes in pulmonary artery pressure and lung fluid balance were observed within 3-h exposure of rabbit lungs to LPS at concentrations of 0.01–100 ng/ml. This low responsiveness is in accord with data obtained in previous studies in buffer-perfused goat (45) and rat lungs (46). It may be due to several factors, including the absence of plasma-borne endotoxin binders such as the LPS-binding protein LBP (3, 47), and the lack of endotoxin-sensitive humoral mediator systems such as the complement cascade. Nevertheless, LPS did provoke significant cytokine generation. Kinetics of TNF appearance (<120 min) and maximum perfusate concentrations of this cytokine (range up to 500 pg/ml; i.e., 5,000 U/ml) corresponded roughly to data reported for intact rabbits

with intravascular endotoxin application (peak TNF concentrations ~2,500 U/ml plasma within 45–100 min; [42]). Lung macrophages are a likely source for TNF (48). It is noteworthy that TNF alone provoked no severe vascular abnormalities and organ injury in our experiments.

When given as sole stimulus, HlyA applied at the very low dose of 1 ng/ml provoked only marginal TxB₂ release and a corresponding moderate increase in pulmonary artery pressure (36). However, dramatically altered responses were observed in lungs primed with LPS, where amplification and acceleration of prostanoid release and pulmonary artery pressure elevation were registered. The potent vasoconstrictor agent thromboxane apparently represented the predominant mediator of the pressor response. Finally this was largely suppressed by cyclooxygenase inhibition and Tx-receptor antagonism. The cellular source of thromboxane in our model is not established, however, lung macrophages represent likely candidates. Preincubation of macrophages with LPS in vitro primes these cells for accelerated and enhanced arachidonate metabolite release in response to subsequent stimulation with opsonized zymosan and the calcium-ionophore A23187, with a half-maximal priming time of ~20 min, and a maximal effect after 50–60 min (6, 11, 49). These features correspond approximately to the characteristics of priming time (obvious effects after ~60 min) and efficacy (acceleration and enhancement of response) in the present study. Further to this, intravascularly sequestered neutrophils, residing in the capillary bed despite extensive buffer-perfusion of the isolated organs (50), may also be targets for synergism between LPS and HlyA. These cells are known to be responsive to both HlyA stimulation (24, 25) and LPS priming (8–10, 15), and neutrophil-derived mediators such as oxygen radicals are potent inductors of lung thromboxane generation (43, 51, 52).

The precise cellular mechanisms of LPS priming are unknown, and many possibilities are under discussion (15, 53, 54). In addition, secondary induction of TNF might add to the priming efficacy of LPS, as suggested from in vitro studies (15, 53, 55). These unresolved questions notwithstanding, it is important to realize that LPS priming can precipitate rapid and total collapse of the lung vascular homeostasis upon challenge with a common membrane-damaging toxin. Pore formers are produced by many other pathogenic microorganisms (31, 32, 56, 57), and their synergism with LPS or other priming agents could be relevant to the pathogenesis and manifestation of organ lesions during severe infections.

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