Brief Definitive Report

Pore-forming Bacterial Toxins Potently Induce Release of Nitric Oxide in Porcine Endothelial Cells

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Summary

Nitric oxide (NO) is believed to play an important role in sepsis-related hypotension. We examined the effects of two pore-forming bacterial exotoxins, *Escherichia coli* hemolysin and *Staphylococcus aureus* α-toxin, on NO formation in cultured porcine pulmonary artery endothelial cells. NO was quantified using a difference-spectrophotometric method based on the rapid and stoichiometric reaction of NO with oxyhemoglobin. Endothelial cyclic guanosine monophosphate levels were also monitored. Both exotoxins increased NO synthesis in endothelial cells in a time- and dose-dependent manner to an extent exceeding that observed with the ionophore A23187 or thrombin. The capacity of exotoxins to induce NO formation may be relevant in patients with severe local or systemic bacterial infections.

V ascular endothelial cells generate nitric oxide (NO) from the terminal guanido nitrogen of L-arginine yielding L-citrulline as a coproduct (1). NO accounts for the biological actions of endothelium-derived relaxing factor, which plays a critical role in regulating vascular tone by stimulating the soluble guanylate cyclase in different target cells (2, 3). Endothelial NO synthase is a NADPH-, Ca^{2+}-, and calmodulin-dependent enzyme that has been found in both cytosolic and particulate cell fractions (3–6).

It has been proposed that enhanced NO synthesis contributes to severe arterial hypotension, which is a hallmark of septic shock (7, 8). This concept evolved primarily from studies that employed L-arginine analogs as inhibitors of NO synthesis (7, 8). Another line of support derived from the demonstration of an immediate release of an NO-like factor from aortic endothelial cells by *Escherichia coli* LPS (9). However, the latter data have not been confirmed (10).

Whether or not proteinaceous bacterial cytotoxins may induce endothelial NO generation is unknown. Staphylococcal α-toxin is a potentially relevant pathogenicity factor that is secreted by most strains of *Staphylococcus aureus* as a hydrophilic single chain polypeptide. After binding to a target lipid bilayer, toxin molecules oligomerize to form amphiphilic ring-shaped hexamers that become partially embedded within the lipid bilayer to generate aqueous transmembrane pores (11, 12). In highly susceptible cells, the pores may act as nonselective

gates for Ca^{2+} ions (11, 13, 14). An increase in intracellular free Ca^{2+} has been linked to toxin-induced generation of prostacyclin in endothelial cells (13) and of leukotriene B4 in granulocytes (15). *E. coli* hemolysin (HlyA) is considered an important virulence factor and hemolysin production is strongly associated with extraintestinal *E. coli* infections (16). Endothelial cells, granulocytes, and renal epithelial cells are highly susceptible targets (17–24). Generation of transmembrane pores is also the mechanism underlying the action of this widespread exotoxin (17, 18, 25). Interest in HlyA is expanding rapidly since this agent is the major cytotoxin produced by *E. coli* (26) and represents the prototype of a large family of toxins that are relevant in human and veterinary medicine (27–32).

In the present study, we examined the ability of *E. coli* HlyA and *S. aureus* α-toxin to induce endothelial NO synthesis. We report that both toxins stimulate endothelial NO synthesis to degrees rivaling and even exceeding those provoked by the Ca^{2+} ionophore A23187 or thrombin. The results identify a potentially important mechanism by which pore-forming toxins may induce hypotension in the course of severe infections or septic shock.

Materials and Methods

Materials. Tissue culture plasticware was obtained from Becton Dickinson (Heidelberg, Germany). Medium 199, FCS, HBSS, PBS, trypsin-EDTA solution, Hepes, and antibiotics were from Gibco (Karlsruhe, Germany). Collagenase (CLS type II) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Cytodex 3 microcarrier beads and Sephadex G-25 column were obtained from Pharmacia (Uppsala, Sweden). Bovine hemoglobin, N^G-nitro-L-arginine (NNA), L-arginine, A23187, and thrombin were from

Sigma (Munich, Germany) and NO gas was purchased from Schuchardt (Munich, Germany). N^G-monomethyl-L-arginine citrate was obtained from Ultrafine Chemicals (Manchester, UK). The ^{125}I-cGMP assay system was from Amersham Buchler (Braunschweig, Germany). LPS from *Salmonella abortus equi* was kindly provided by Dr. C. Galanos (Max-Planck-Institute, Freiburg, Germany).

Preparation of E. coli HlyA and S. Aureus α-toxin. LPS-depleted HlyA was prepared as described (19). The endotoxin content of the preparation was reduced to <3 ng of LPS/μg protein. The hemolytic titer was assessed directly before use and expressed in hemolytic units (HU/ml) (17, 18); 1 HU/ml HlyA corresponded to ~100 ng protein/ml. Toxin activity was lost rapidly at 37°C ("aged" toxin) (17, 18). This well-known property of the toxin was exploited to exclude possible effects of contaminating LPS (19, 33). *S. aureus* α-toxin was purified as described by Bhakdi et al. (34).

Preparation of Endothelial Cells. Pulmonary artery endothelial cells were isolated from freshly slaughtered pigs by exposure to 0.1% collagenase for 12–15 min. Cells were characterized, maintained, and dispersed as previously described (13, 33, 35). All studies (quantification of NO or cGMP, lactate dehydrogenase [LDH] release, use of inhibitors) were performed on endothelial cells in their third passage.

Endothelial Cells on Microcarrier Beads and Experimental Protocol. Third-passage cells were grown in medium 199/10% FCS on Cytodex 3 microcarrier beads for 5–8 d until confluence (36). Cells on 5 ml packed beads were then washed several times and preincubated with 10 ml PBS containing 4 μM oxyhemoglobin for 30 min. A 1-ml aliquot was taken, spun at 500 *g* for 5 min, and the supernatant used for NO determination (time 0, control). Stimuli (*E. coli* HlyA, staphylococcal α-toxin, A23187, or thrombin) were added, and aliquots of the bead suspension were taken at different time points for NO determinations. In some experiments, cells were preincubated with different concentrations of NNA for 30 min.

Determination of NO. Endothelial NO synthesis was quantified using a difference-spectrophotometric method based on the rapid and stoichiometric reaction of NO with oxyhemoglobin to yield methemoglobin and nitrate as described by Feelisch and Noack (37). For this, the extinction difference between the absorption maximum and the isobestic point of oxyhemoglobin versus methemoglobin (401 and 411 nm) was recorded using a spectrophotometer (Uvikon, Munich, FRG) (38). For preparation of oxyhemoglobin, bovine hemoglobin was dissolved in water, equilibrated with oxygen, reduced with a molar excess of sodium dithionite, and purified on Sephadex G-25 columns (38). Methemoglobin was prepared by treating hemoglobin with a 10-fold molar excess of potassium ferricyanide, before gel filtration (38). NO biosynthesis was also monitored by determining endothelial cGMP levels. Confluent cell monolayer in 24-well plates were washed and stimulated in HBSS/Hepes for 30 s–30 min in the presence of the unselective phosphodiesterase inhibitor isobutyl-methylxanthine. Then cells were extracted twice with 0.5 ml ice-cold 65% ethanol. Extracts were evaporated under a stream of nitrogen at 60°C and dissolved in assay buffer. Aliquots of the extracts and of the standards were acetylated by addition of acetic anhydride and triethylamine (1:2) to enhance the sensitivity of cGMP detection (39).

Determination of LDH. LDH release from cell monolayers cultured in 24-well plates was used as a marker of overt cytotoxicity. The medium was removed and centrifuged at 8,000 *g* for 2 min. LDH activity in the supernatant was determined by the colorimetric measurement of the reduction of sodium pyruvate in the presence of NADH as described (40). Enzyme release was expressed

as the percentage of total enzyme activity liberated from endothelial cells in the presence of 100 μg/ml mellitin (35, 40).

Statistical Methods. Depending on the number of groups (A) and the number of different time points studied (B), data of Figs. 1 and 3 were analyzed by an A × B analysis of variance (ANOVA). A one-way ANOVA was used for data of Fig. 2. *p* <0.05 was considered significant (41).

Results and Discussion

Unstimulated endothelial cells produced NO at a rate of 12 ± 3.9 pmoles/min/ml packed beads. This rate of spontaneous NO synthesis was comparable to that reported by Kelm et al. (38). *E. coli* HlyA dose (0.05–1.0 HU/ml) and time dependently increased NO generation in porcine pulmonary artery endothelial cells (Fig. 1 *A*). After 30 min, 1.83 ± 0.145 nmoles NO/ml packed beads were generated (3.2 ± 0.22 nmoles NO after 60 min) through the action of 1 HU/ml HlyA. Staphylococcal α-toxin also induced endothelial NO synthesis in a dose- and time-dependent manner. 1.31 ± 0.065 nmoles NO/ml packed beads were generated after 30 min in the presence of 1 μg/ml staphylococcal α-toxin (Fig. 1 *B*). In this system, staphylococcal α-toxin and HlyA were thus as potent as 10 μM A23187 (Fig. 1 *C*). Thrombin (2 U/ml) increased NO synthesis to maximally 0.72 ± 0.06
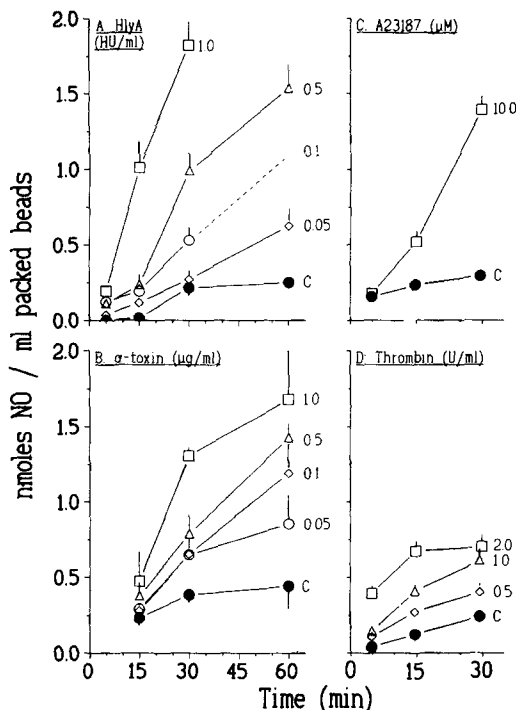

Figure 1. Stimulation of NO generation in pulmonary endothelial cells by *E. coli* HlyA, *S. aureus* α-toxin, the ionophore A23187, and thrombin. NO was quantified by monitoring the oxidation of hemoglobin (see Materials and Methods). 0.05 HU/ml HlyA (*A*) and 0.05 μg/ml staphylococcal α-toxin (*B*) or more significantly increased endothelial NO synthesis. The effects of 10 μM A23187 (*C*) and 1 U/ml thrombin (*D*) were also significant above baseline NO formation. Data presented are mean ± SE of six (*A* and *B*) and four (*C* and *D*) separate experiments.

nmoles/ml packed beads The thrombin effect reached a plateau after 15 min (Fig. 1 D). From Fig. 1, the following rank order of NO-inducing potency emerged: E. coli HlyA > S. aureus α-toxin = A23187 > thrombin. Incubation of endothelial cells with 100 ng/ml LPS for 1 h did not increase NO synthesis (data not shown). "Aged" HlyA (toxin kept at 37°C for 6 h) also did not stimulate NO formation.

NNA was used as an arginine analog to confirm that hemoglobin oxidation was NO related. 2.5 and 10 μM NNA reduced A23187-induced NO synthesis by 75 and 95%, respectively.

cGMP content in endothelial cells was determined as an indirect parameter of NO synthesis (Fig. 2). Both toxins induced a rapid increase in endothelial cGMP levels. HlyA at the very low concentrations of 0.05–0.1 HU/ml was again observed to be as potent as the Ca^{2+} ionophore A23187, inducing a 10-fold increase in cGMP concentrations within 5 min. S. aureus α-toxin at 0.5 μg/ml also induced a marked, approximately fivefold increase in cGMP. Thrombin again was the weakest stimulus, inducing only an approximately twofold cGMP increase. NNA abrogated the observed effects in all cases (Fig. 2). It is of interest that cGMP levels did not increase in the presence of hemoglobin, suggesting that endothelial cell–derived NO acted in a paracrine (not autocrine) fashion on endothelial guanylate cyclase (Fig. 2).

Figure 3. Effect of Ca^{2+} depletion on stimulation of NO synthesis in pulmonary endothelial cells by staphylococcal α-toxin and the ionophore A23187. In the absence of extracellular Ca^{2+} (filled symbols) staphylococcal α-toxin (0.5 μg/ml)-induced NO synthesis was abrogated. Under these conditions A23187-related NO formation was also strongly reduced and only slightly above baseline at 30 min (B). Data presented are mean ± SE of four separate experiments.

To further characterize exotoxin-related stimulation of NO synthesis in endothelial cells, experiments were performed in the absence of extracellular Ca^{2+}. The effect of staphylococcal α-toxin on endothelial NO formation was abrogated under these conditions, suggesting that an influx of extracellular Ca^{2+} is important for the action of this toxin (Fig. 3 A). A23187-related NO synthesis was also strongly reduced and only slightly above baseline at 30 min (Fig. 3 B).

LDH release was not enhanced in A23187- and thrombin-stimulated cells. Slight cytolysis occurred in endothelial cells after stimulation with maximal HlyA or staphylococcal α-toxin concentrations at 30–60 min. 1 HU/ml HlyA increased LDH release to 12.3 ± 1.7% in 30 min, and 1 μg/ml staphylococcal α-toxin to 13.2 ± 4.3% in 60 min over their baseline controls. Thus, stimulation of NO formation occurred at toxin concentrations well below those required to cause overt cytolysis.

Salvemini et al. (9) reported on an immediate release of a NO-like factor from bovine aortic endothelial cells induced by high (10 μg/ml) concentrations of E. coli LPS. Although this was later contested by Myers et al. (10), the possibility remained that NO synthesis observed in our experiments may have been due to contaminating endotoxin. However, our HlyA preparations contained <3 ng LPS/μg protein so that LPS concentrations were well below 2–5 ng/ml at the highest HlyA concentrations employed. Incubation of cells with up to 100 ng/ml endotoxin did not increase endothelial NO synthesis within 1 h. Furthermore, "aged" HlyA which had lost its cytotoxic activity also no longer provoked NO release. These data therefore virtually exclude a direct effect of contaminating LPS in our experiments. They do not exclude a possible synergistic interaction between HlyA and low LPS concentrations. That a pore-forming proteinaceous toxin alone is capable of inducing NO release from endothelial cells was apparent from the results obtained with S. aureus α-toxin, which was derived from a gram-positive (LPS-free) organism.

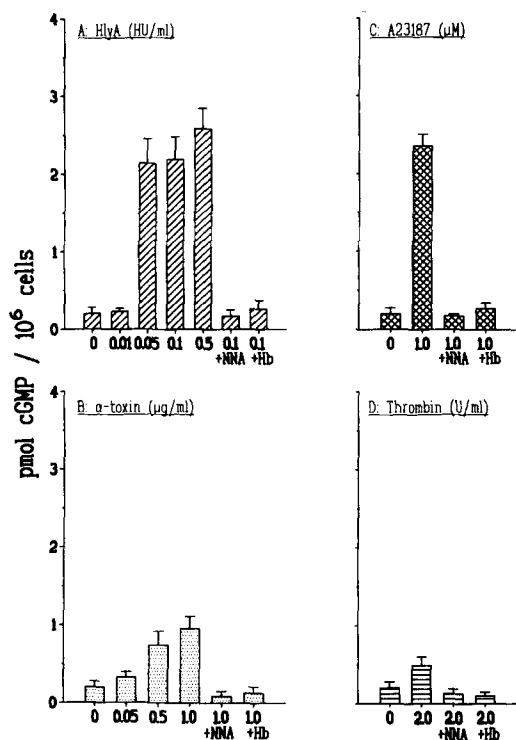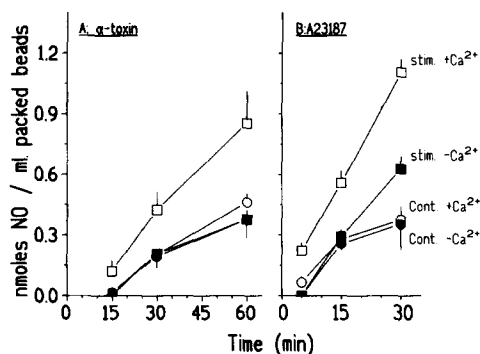Further experiments are required to delineate the mecha-

Figure 2. cGMP content of pulmonary artery endothelial cells was used as an indirect parameter of endothelial NO synthesis. cGMP-levels were increased significantly by >0.05 HU/ml HlyA, >0.5 μg/ml α-toxin, 1 μM A23187, or 2 U/ml thrombin. In the presence of 10 μM NNA or hemoglobin endothelial cGMP concentrations were reduced to basic levels. Data are given as pmol cGMP/10^6 endothelial cells 5 min after stimulation and are presented as mean ± SE of three to eight separate experiments.

nisms by which bacterial exotoxins trigger metabolic pathways in endothelial cells. Ca^{2+} influx is an obvious candidate and the initial experiments reported here for staphylococcal α-toxin are in accord with this basic idea. Experiments with HlyA are underway, but they are impeded by the fact that HlyA preparations contain high concentrations of Ca^{2+} and that HlyA requires Ca^{2+} in order to bind to cells (42, 43).

These unresolved questions notwithstanding, the present study shows that proteinaceous exotoxins released in the vicinity of vascular endothelium may potently and rapidly provoke NO release, thus providing a novel pathway leading to hypotension and organ failure.

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