Clostridium perfringens Epsilon-Toxin Increases Permeability of Single Perfused Microvessels of Rat Mesentery

R. H. Adamson,^{1*} J. C. Ly,¹ M. Fernandez-Miyakawa,² S. Ochi,³ J. Sakurai,³
F. Uzal,² and F. E. Curry¹

Department of Physiology and Membrane Biology, School of Medicine, University of California, Davis, One Shields Avenue, Davis, California 95616¹; California Animal Health and Food Safety Laboratory, University of California, Davis, 105 W. Central Avenue, San Bernardino, California 92408²; and Department of Microbiology, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan³

Received 16 December 2004/Returned for modification 17 January 2005/Accepted 10 February 2005

Epsilon-toxin, the primary virulence factor of *Clostridium perfringens* type D, causes mortality in livestock, particularly sheep and goats, in which it induces an often-fatal enterotoxemia. It is believed to compromise the intestinal barrier and then enter the gut vasculature, from which it is carried systemically, causing widespread vascular endothelial damage and edema. Here we used single perfused venular microvessels in rat mesentery, which enabled direct observation of permeability properties of the in situ vascular wall during exposure to toxin. We determined the hydraulic conductivity (L_p) of microvessels as a measure of the response to epsilon-toxin. We found that microvessels were highly sensitive to toxin. At $10 \mu\text{g ml}^{-1}$ the L_p increased irreversibly to more than 15 times the control value by 10 min. At $0.3 \mu\text{g ml}^{-1}$ no increase in L_p was observed for up to 90 min. The toxin-induced increase in L_p was consistent with changes in ultrastructure of microvessels exposed to the toxin. Those microvessels exhibited gaps either between or through endothelial cells where perfusate had direct access to the basement membrane. Many endothelial cells appeared necrotic, highly attenuated, and with dense cytoplasm. We showed that epsilon-toxin, in a time- and dose-dependent manner, rapidly and irreversibly compromised the barrier function of venular microvessel endothelium. The results conformed to the hypothesis that epsilon-toxin interacts with vascular endothelial cells and increases the vessel wall permeability by direct damage of the endothelium.

One of the most potent of the clostridial toxins, epsilon-toxin of *Clostridium perfringens*, has been named a class B select agent of the Centers for Disease Control and Prevention and a category B priority agent of the National Institute of Allergy and Infectious Diseases. While newly regarded as a potential bioterrorism tool, epsilon-toxin has long been recognized as an important cause of mortality in livestock, particularly sheep and goats, where it is the primary virulence factor of *C. perfringens* type D and leads to an often-fatal enterotoxemia. *C. perfringens* type D can be a normal inhabitant of the intestine of sheep and several other animal species, but when large amounts of easily fermentable carbohydrates pass into the intestine, generally due to sudden changes in diet, this microorganism proliferates and produces large quantities of epsilon-toxin. Although little evidence is available in this regard, it is generally accepted that epsilon-toxin compromises the intestinal barrier and is then taken up by the vasculature of the gut, from which it is spread systemically. The effects of injecting epsilon-toxin intravenously have been studied in sheep (7), mice (10), goats (38), rats (11), and cattle (39). The toxin thus administered produces increased vascular permeability in many tissues, the most significant effects of which are acute pulmonary and cerebral edema, which is associated with vascular endothelial injury demonstrable with transmission electron microscopy (13). Thus, the toxemia has been assumed to

directly cause widespread damage to the vascular endothelium, although no definitive proof has been produced about the direct action of epsilon-toxin on endothelial cells.

Of the many cultured cells that have been tested for sensitivity to epsilon-toxin, only Madin-Darby canine kidney (MDCK) cells and Caucasian renal leiomyoblastoma (G-402) cells are sensitive enough for useful investigation of the toxin (4, 32, 40). The MDCK cells are about 100-fold more sensitive than the G-402 cells and have been used in most cultured cell studies of epsilon-toxin. In MDCK cells, epsilon-toxin was found in high-molecular-weight complexes and induced increased membrane permeability to ions and small hydrophilic solutes (33). It forms an aqueous anion-selective pore permeable to solutes up to 1 kDa (35). Analysis of labeled constructs derived from epsilon-toxin clearly demonstrated that it forms heptamers when incubated with rat brain synaptosomal membranes and that cleavage of a C-terminal peptide is essential for oligomerization (26). Also in MDCK cells, epsilon-toxin associates with apical membrane in preference to basolateral membrane and forms a heptameric pore associated with detergent-resistant domains (27, 34). While it has relatively low amino acid sequence identity (ca. 14%) to the heptameric pore-forming toxin aerolysin from *Aeromonas hydrophila*, it does have three-dimensional structural similarity to aerolysin, one of a family of toxins known as β -pore-forming toxins (8). Epsilon-toxin also has some amino acid sequence identity to a family of mosquitocidal toxins typified by Mtx2 and Mtx3 of *Bacillus sphaericus* (21% and 24% identity, respectively) (9, 22, 37). Because only some cultured cells are highly sensitive to the toxin it has been suggested that a specific host receptor is

* Corresponding author. Mailing address: Department of Physiology and Membrane Biology, University of California, Davis, One Shields Avenue, Davis, CA 95616. Phone: (530) 752-2180. Fax: (530) 752-5423. E-mail: rhadamson@ucdavis.edu.

essential for binding and pore formation, but no specific receptor molecule has been found. A recent study demonstrated that experimental development of tolerance to epsilon-toxin by MDCK cells was associated with loss of expression of a group of membrane proteins, lending further support to the hypothesis that host proteins mediate epsilon-toxin binding and toxicity (4). Because of the assumed association of epsilon-toxin with vascular endothelium, development of an endothelial model is highly desirable. However, cultured aortic endothelial cells (goat, sheep, and cattle) failed to show any morphological response to epsilon-toxin with up to 48 h of exposure, even at concentrations much higher than were toxic for MDCK cells (40). The latter observation brought into question whether the *in vivo* toxicity of epsilon-toxin is due to direct action of the toxin with vascular endothelium.

Because the cultured endothelial cells were not morphologically affected by epsilon-toxin, in this study we have used single perfused microvessels in rat mesentery, which enable direct observation of permeability properties of the vascular wall during exposure to toxin under controlled conditions. While the effects of epsilon-toxin in tissue have been investigated using morphological and histochemical techniques to demonstrate accumulations of fluid and labeled proteins, there are no reports in the literature of biophysical permeability coefficients determined from directly measured fluxes and controlled driving gradients. Moreover, by presenting the toxin directly to the endothelium within an artificial perfusate, the technique enables investigation of the endothelial response in the absence of potential systemic mechanisms or interactions with blood plasma. Variations of the single perfused microvessel model have been used to investigate vascular permeability coefficients regarding convective water flow and permeability of both small and large solutes (19, 24, 25), regulation of inflammatory mechanisms (3, 16), ultrastructure of the endothelial cleft (2), and regulation of the adherens junction complex (1, 42). Here we determine the hydraulic conductivity coefficient (L_p) as a measure of the response to epsilon-toxin. We demonstrate that microvessels of the rat mesentery are highly sensitive to epsilon-toxin, and we describe an experimental system in which to investigate the direct action of bacterial toxins with vascular endothelium.

MATERIALS AND METHODS

Animal preparation. Experiments were carried out on rats (male, Sprague-Dawley, 350 to 450 g; Hilltop Laboratory Animals, Inc.) anesthetized with pentobarbital (65 mg kg⁻¹ body weight given subcutaneously). Anesthesia, assessed by toe-pinch reflex, was maintained by giving additional pentobarbital (subcutaneous, 3 mg per dose) as needed. At the end of experiments, animals were euthanized with pentobarbital overdose. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of California, Davis. Each rat was placed on a heating pad to maintain normal body temperature. A midline surgical incision was made in the abdominal wall, and the gut gently was taken from the abdominal cavity to spread the mesentery over a quartz pillar for transillumination and observation on the fixed stage of an inverted microscope. The upper surface of the mesentery was continuously superfused with Ringer's solution maintained at 35°C to 37°C during preparation and experimentation. Because our present techniques are not suited for use of vessels smaller than about 15 μ m in diameter, we have not investigated the properties of true capillaries (diameter of ca. 6 μ m). Therefore, experiments were performed on straight nonbranched segments of venular microvessels typically 25 to 40 μ m in diameter and usually between 700 and 900 μ m in length. Prior to cannulation, all vessels selected for experiments had brisk blood flow and were free of leukocytes sticking or rolling on the vessel wall.

Measurement of L_p of the microvessel wall. All measurements were based on the modified Landis micropertusion technique, which enables measurement of the volume flux of water across the wall of a microvessel due to a known pressure difference between the lumen and the interstitium. The technique and its assumptions and limitations have been evaluated in detail (2, 24). Briefly, each vessel was cannulated by the tip of a glass micropipette filled with a physiological perfusate additionally containing flow marker erythrocytes (ca. 4%, vol/vol). The micropipette was positioned in the vessel to achieve free downstream perfusion with no leak to the upstream direction or to the tissue. The rear of the micropipette was in communication with a water manometer used to set the hydrostatic pressure and drive flow from the micropipette through the cannulated vessel. To measure the initial transcapillary water flow per unit area of the capillary wall (J_v/A), a glass microoccluder was lowered onto the microvessel up to 900 μ m from the cannulation site, with the hydraulic pressure set at predetermined values of 30 to 60 cm H₂O. When the vessels were occluded, the intravascular hydraulic pressure was equal to that of the manometer, and the motion of flow marker erythrocytes was recorded on videotape for later analysis. Microvessel L_p was calculated as the slope of the relation between J_v/A and applied hydraulic pressure. For most experiments, J_v/A was estimated from each of several occlusions using one hydraulic pressure, with the assumption that the net effective pressure determining fluid flow was equal to the applied hydraulic pressure minus 3.6 cm H₂O, the approximate osmotic pressure contributed by bovine serum albumin (BSA) at 10 mg ml⁻¹. Experimental reagents (e.g., epsilon-toxin) were added to the perfusate in the respective experiments and delivered to the vascular lumen via the micropipette continuously during L_p measurement. Changes in perfusate were accomplished by withdrawing the initial micropipette and replacing it with a second micropipette filled with new perfusate solution of the appropriate composition.

Preliminary experiments were designed to determine the effective osmotic pressure ($\Delta\pi_{\text{eff}}$) of albumin (30 mg ml⁻¹) in the perfusate. Before each measurement the hydraulic pressure in the vessel was held at 60 cm H₂O for 30 s by partial downstream occlusion to establish a high-filtration steady state in which the BSA concentration on the downstream side of the selectivity barrier was washed down to a minimal level, thus maximizing the effective oncotic pressure difference (24, 25). J_v/A was measured by monitoring the motion of flow marker erythrocytes during brief (5 to 8 s) full occlusions. With 60 cm H₂O hydraulic pressure, erythrocytes moved toward the occlusion site as fluid moved out of the vessel. Measurements of J_v/A at microvessel pressure of 10 cm H₂O were made after setting up steady-state filtration at 60 cm H₂O, occluding, and rapidly (<0.5 s) switching the applied hydraulic pressure to 10 cm H₂O. Reabsorptive water movement was seen as marker erythrocytes moved away from the occlusion site toward the cannulation site as fluid moved into the vessel. Three to five measurements were made at each pressure, yielding a control relationship between J_v/A and applied hydraulic pressure. The slope of the relationship is the hydraulic conductivity (L_p), and the intercept on the pressure axis is $\Delta\pi_{\text{eff}}$ of the serum albumin. Our test solutions of BSA (10 or 30 mg ml⁻¹) had protein osmotic pressures of 3.6 and 14 cm H₂O at 37°C (calculated from published equations [23]).

Experimental protocols. Each vessel was initially perfused with a control solution containing BSA at 10 mg ml⁻¹ in Ringer's solution (except preliminary experiments as indicated). Usually between 5 and 10 occlusions at 50 cm H₂O over 15 to 20 min were used to establish a control L_p . Then, the first pipette was removed and a second pipette containing the test solution was introduced at the same cannulation site. Multiple occlusions over the subsequent 15 to 120 min enabled estimation of L_p during exposure to the test solution. Occlusions were made every 20 to 30 s during the first 5 min of test perfusion to check for rapid initial change in J_v/A and then made less frequently (three to five occlusions every 5 to 10 min) for the remainder of the experiment.

Statistics. We tested for differences from basal L_p due to the perfusion with epsilon-toxin by comparing the averaged L_p in each experimental group with the corresponding time-matched value found in a control (no-toxin) group of vessels. Because basal L_p distribution is non-Gaussian, we used the nonparametric Mann-Whitney statistic and assumed significance for a *P* value of <0.05. Throughout, descriptive statistics are reported as the mean $\pm$ the standard error of the mean (SEM).

Ultrastructure. To investigate the structural nature of the response to toxin, four vessels exposed to epsilon-toxin (two using 3 μ g ml⁻¹ and two using 10 μ g ml⁻¹) were fixed after a substantial rise in L_p (25 to 40 min of toxin exposure). Two other vessels were perfused with vehicle solution (control vessels) for a comparable time and also inspected using the following methods. After the final determination of J_v/A , fixation for electron microscopy was started by dripping ice-cold glutaraldehyde (3% in 0.1 mM cacodylate buffer) on the mesentery. This was done while vessels were still perfused and occluded to ensure that the final

perfusion conditions and geometry were maintained during fixation. After 1 to 2 min the perfusion pipette was removed while immersion fixation continued. A diagram of the area enabled later identification of the perfused vessel segment. After 10 min in situ fixation, tissue was cut away from the animal and glutaraldehyde fixation continued on ice for a total of 1 h before changing to OsO_4 (1% in veronal acetate, pH 7.2) for 1 h. Tissue was treated with tannic acid (0.5% in maleate buffer, pH 6) for 30 min to enhance membrane staining and placed in uranyl acetate (2% in maleate buffer, pH 6) overnight. After dehydration in a graded series of acetone, the tissues were flat embedded in epoxy resin. Sections (50 nm thick) from transversely sectioned vessels were collected on Formvar-coated slots and were stained with uranyl acetate and lead citrate. They were examined using transmission electron microscopy (TEM) with a Philips CM120 at 80 kV.

Solutions and reagents. Mammalian Ringer's solution was composed (in mM) of 132 NaCl, 4.6 KCl, 2 CaCl_2 , 1.2 MgSO_4 , 5.5 glucose, 5.0 NaHCO_3 , and 20 HEPES and Na-HEPES. The ratio of acid-HEPES to Na-HEPES was adjusted to achieve pH 7.40 to 7.45 for Ringer's solutions. All perfusates were mammalian Ringer's solution additionally containing BSA (A4378; Sigma-Aldrich) at 10 or 30 mg ml^{-1} as indicated.

Epsilon-prototoxin was prepared from an overnight culture of *C. perfringens* type D (strain NCTC 8346) in Trypticase-yeast-glucose medium, under anaerobic conditions at 37°C. The overnight cultures were centrifuged at 10,000 rpm for 30 min at 4°C, and the supernatant containing epsilon-toxin was saved for toxin purification. The toxin was then precipitated by ammonium sulfate. Two columns were prepared with DEAE and CM Sepharose (Pharmacia, Sweden), respectively, equilibrated in 10 mM Tris, pH 7.5. The toxin was applied to the DEAE column, and the effluent was monitored at 220 nm. The initially eluted peak was saved and applied to the CM column. Again the effluent was monitored at 220 nm, and the first peak was collected, dialyzed against distilled water, and freeze-dried. This product was shown to contain pure epsilon-prototoxin when tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Prior to its use in these experiments, the prototoxin was reconstituted in Ringer's and activated by incubation at 37°C during 30 min with 0.1% trypsin (Sigma).

To further establish specificity of the L_p response to toxin, we tested the effectiveness of an epsilon-toxin neutralizing mouse monoclonal antibody (immunoglobulin G; 5B7) provided by Paul Hauer (Center of Veterinary Biologics, Ames, Iowa). The antibody (final concentration, 0.13 mg ml^{-1}) was added to perfusion solution containing epsilon-toxin (3 $\mu\text{g ml}^{-1}$) and incubated for 30 min at room temperature before use. The effect on microvascular L_p was measured and compared to the time-matched set of measurements made with epsilon-toxin in the absence of antibody.

RESULTS

Epsilon-toxin increases microvascular L_p . We perfused single microvessels of rat mesentery with solutions containing epsilon-toxin to test for increased L_p as an indication of endothelial cell intoxication. In a few preliminary experiments the perfusate contained 30 mg ml^{-1} BSA, enabling determination of the effective protein osmotic pressure difference across the vessel wall. The data from a representative experiment illustrate that during control measurement in the absence of toxin the L_p (slope of the relation between J_v/A and applied pressure) was low and the effective protein osmotic pressure (intercept on the pressure axis) was between 10 and 20 $\text{cm H}_2\text{O}$ (Fig. 1). After removing the initial pipette and perfusing with a second pipette containing toxin (10 $\mu\text{g ml}^{-1}$), the L_p increased over time and the effective protein osmotic pressure fell to near zero. Both changes are consistent with loss of endothelial barrier function. This initial technique required multiple measurements, typically taking several minutes at various pressures. When permeability changed rapidly, it was difficult to make estimates of the intercept on the pressure axis. Therefore, in remaining experiments the BSA concentration was 10 mg ml^{-1} and estimates of L_p were made using one applied pressure (usually 50 or 60 $\text{cm H}_2\text{O}$) as described in

FIG. 1. Representative data from a single experiment showing transmural fluid flux (J_v/A) against applied hydraulic pressure. During control perfusion in the absence of toxin, the L_p was $2.5 \times 10^{-7} \text{ cm} \times (\text{s} \times \text{cm H}_2\text{O})^{-1}$ and $\Delta\pi_{\text{eff}}$ was near 18 $\text{cm H}_2\text{O}$. After 10 min of perfusion with toxin, the L_p increased to $5.5 \times 10^{-7} \text{ cm} \times (\text{s} \times \text{cm H}_2\text{O})^{-1}$ and $\Delta\pi_{\text{eff}}$ had fallen to near zero. By near 20 min of toxin exposure, the L_p had risen to $13.5 \times 10^{-7} \text{ cm} \times (\text{s} \times \text{cm H}_2\text{O})^{-1}$ and $\Delta\pi_{\text{eff}}$ remained negligible.

Materials and Methods. The latter technique better enabled our following of rapid L_p changes.

A representative experiment shows the increase in L_p with time associated with toxin exposure (Fig. 2A). Perfusion with vehicle control solution for 30 min established the control L_p of $0.3 \times 10^{-7} \text{ cm s}^{-1} \text{ cm H}_2\text{O}^{-1}$. The initial micropipette was removed, and the vessel was cannulated a second time and perfused with toxin (3 $\mu\text{g ml}^{-1}$) for the next 40 min (Fig. 2A). During the first 5 min of perfusion with toxin, there was no apparent change in L_p . However, within 10 min of exposure to

FIG. 2. Representative L_p data from two individual microvessels. (A) After perfusion with control (no toxin) solution for 30 min, the vessel was recannulated with epsilon-toxin (3 $\mu\text{g ml}^{-1}$). After about 5 min of exposure to epsilon-toxin the L_p of this vessel increased steadily during 40 min of perfusion. (B) Data from a separate experiment illustrate that perfusion with control solution (no toxin) does not induce a change in L_p .

FIG. 3. (A) Dose-response relationship between L_p and toxin concentration determined in four different groups of venular microvessels. No response was found with up to 90 min of perfusion at 0.3 $\mu\text{g ml}^{-1}$ toxin (lowest dose tested). At 10 $\mu\text{g ml}^{-1}$ toxin some vessels responded within the first minute. The threshold concentration to measurably increase L_p within 60 to 90 min was 1 $\mu\text{g ml}^{-1}$. *, for both 10 and 3 $\mu\text{g ml}^{-1}$ the L_p values were greater than time-matched controls at all times ($P < 0.05$, Mann-Whitney test); †, for 1 $\mu\text{g ml}^{-1}$ the L_p was greater than control at 50 min and longer ($P < 0.05$, Mann-Whitney test); ‡, the group treated with 0.3 $\mu\text{g ml}^{-1}$ was not different from control at any time ($P > 0.05$, Mann-Whitney test). (B) Average L_p in vessels perfused without toxin shows no change over 90 min. Error bars are smaller than symbols and do not appear for this group. Note the different vertical scale. The number of vessels in each group (mean $\pm$ SEM) is shown.

epsilon-toxin the L_p increased to about $2 \times 10^{-7} \text{ cm s}^{-1} \text{ cm H}_2\text{O}^{-1}$. It continued to increase and was above $15 \times 10^{-7} \text{ cm s}^{-1} \text{ cm H}_2\text{O}^{-1}$ by 40 min of toxin exposure. Representative data from a vessel perfused for 90 min without toxin illustrate that the perfusion technique alone does not increase L_p (Fig. 2B). Averaged data from groups of experiments are described next.

To test the concentration dependence of the sensitivity to epsilon-toxin in the microvasculature, the L_p response to toxin over time was tested in four different groups of vessels (Fig. 3A). A separate group of vessels perfused without toxin for 90 min demonstrated that L_p did not change in that group ($n = 15$) (Fig. 3B). The L_p values from that group were used as time-matched control values for the toxin-perfused vessels. At the lowest toxin concentration (0.3 $\mu\text{g ml}^{-1}$) no increase in L_p was observed for up to 90 min ($n = 7$). At 10 $\mu\text{g ml}^{-1}$ the L_p increased rapidly, on average to more than 15 times the control value within 10 min, and was significantly greater than the time-matched control group at all times ($P < 0.05$, Mann-Whitney). At intermediate concentrations the response to epsilon-toxin exposure took longer to develop and the mean values of L_p did not reach as high. The threshold concentration for a measurable L_p response within 60 min was 1 $\mu\text{g ml}^{-1}$

FIG. 4. Demonstration of toxin neutralization with monoclonal antibody 5B7. In vessels perfused with toxin additionally treated with neutralizing antibody there was no change in L_p , showing that the L_p response is dependent on the toxin. The number of vessels in each group (mean $\pm$ SEM) is shown.

(significantly greater than time-matched control group at 50 min and longer). In two experiments, after the L_p had significantly increased during epsilon-toxin exposure, each vessel was recannulated with the control perfusate and L_p measurements were made for 20 min to test for recovery from the epsilon-toxin. The L_p in these vessels did not return toward control; the effect of epsilon-toxin to increase L_p was irreversible.

Antibody neutralization of the toxin effect on L_p . A separate group of vessels were perfused with solution containing a neutralizing monoclonal antibody (0.13 mg ml^{-1}) to epsilon-toxin in addition to epsilon-toxin (3 $\mu\text{g ml}^{-1}$). In perfusions lasting up to 40 min, the L_p in these vessels did not increase. L_p measured over time is shown in comparison with the mean response to the same concentration of toxin alone in Fig. 4. Over this time the antibody completely prevented the increase in L_p induced by toxin in the absence of antibody.

Ultrastructure of microvessels exposed to toxin. Two vessels of the group perfused with 3 $\mu\text{g ml}^{-1}$ epsilon-toxin and two of the group perfused with 10 $\mu\text{g ml}^{-1}$ epsilon-toxin were examined with TEM. The two vessels exposed to 3 $\mu\text{g ml}^{-1}$ toxin (25 and 40 min, respectively) reached an L_p about 5 times the control value just before fixation; in the two vessels exposed to 10 $\mu\text{g ml}^{-1}$ epsilon-toxin (25 and 35 min, respectively), L_p had increased to 13 and 24 times the control L_p when fixed. Features consistent with direct endothelial damage were observed in all epsilon-toxin-treated vessels (Fig. 5). Most endothelial cells were very attenuated, showing loss of cytoplasm, although the cytoplasmic membrane appeared intact in most cases (Fig. 5A, B, C, and G). Different degrees of detachment from the basement membrane were also observed. A few cells were also fragmented, with some of the fragments remaining attached to the basement membrane. Thus, in places the endothelium was discontinuous, exhibiting gaps either between or through the endothelial cells (Fig. 5A, B, D, and E). Near one such gap the subendothelial space between the basement membrane and pericyte was expanded and filled with flocculent material, possibly protein from disrupted cells (Fig. 5A and B). Sections of the basement membrane were exposed to the vascular lumen, although the basement membrane in these areas and else-

FIG. 5. TEM images of vessels perfused with epsilon-toxin, illustrating a range of damage to endothelial cells and pericytes. (A) Low-magnification overview shows gap in endothelium, enlarged vacuole (vac), and high-density nuclear chromatin in an endothelial cell (nuc). Pericyte (peri) is separated from basement membrane, and the space is filled with flocculent material (floc). (B) Detail of panel A shows irregular endothelial surface (is) with regions separated from basement membrane (bm). Extreme density of pericytes also suggests intoxication. Endothelial junctional complex (jc) appears intact. (C) Highly attenuated and electron-dense endothelial cell (ec1) contrasts with relatively normal appearance of second endothelial cell (ec2) and nearby mesothelial cell (mes). Collagen (col) and elastic fibers (el) appear normal. (D) Large region over which endothelium is discontinuous, with only cell debris remaining loosely adherent to exposed basement membrane. (E) Exposed basement membrane and nearby endothelium that appears irregularly bulbous and lacking typical fine structure. (F) Swollen segment of endothelial cell (luc) with vesicular structures on an unusually electron lucent background. Nearby junctional complexes appear intact. (G) Segment of an endothelial cell with two regions empty of cytoplasm such that the luminal cytoplasmic membrane has collapsed onto the abluminal membrane (arrows). er, endoplasmic reticulum. Inset at higher magnification clearly shows double membrane layers (small arrows) and an apparently intact endothelial tight junction (tj).

where appeared intact. Some of the pericytes were also densely stained and highly attenuated (Fig. 5A and B). Most endothelial cells had angular shapes, and the cytoplasmic membrane presented an irregular outline, with numerous indentations and a few cytoplasmic blebs consisting of flocculent relatively electron-lucent material devoid of organelles and surrounded by cytoplasmic membrane. Most endothelial cells showed a diffusely electron-dense cytoplasm in which it was impossible to distinguish any cytoplasmic detail. A few endothelial cells, however, showed a clear cytoplasm in which dilated cisterns of endoplasmic reticulum were observed. Very few ribosomes were seen attached to the endoplasmic reticulum membranes. A few membrane-bound vacuoles with myelinic figures in front of a clear background were also seen in the cytoplasm of some cells. The nuclei of some endothelial cells were shrunken and showed large clumps of electron-dense chromatin. A few cells showed diffuse dense chromatin occupying the entire nucleus. Nuclear membranes were unaffected in most cases. Endothelial cells of control vessels perfused for comparable times with vehicle solutions in the absence of toxin did not show ultrastructural abnormalities, such as gaps in the endothelium or necrotic characteristics (Fig. 6). Specifically, the endothelium was in contact with basement membrane and formed a continuous intimal layer with intact junctional complexes. The peripheral cytoplasm was of moderate electron density and was relatively uniform in thickness (0.1 to 0.2 μm).

DISCUSSION

We set out to test the specific hypothesis that epsilon-toxin would increase the hydraulic permeability of microvessels in the rat mesentery and to answer the more general question of whether the single perfused vessel model could be used to investigate epsilon-toxin. The data demonstrated a time- and dose-dependent irreversible increase in convective water flux across the vessel walls in response to exposure to activated epsilon-toxin. Many endothelial cells from toxin-treated vessels appeared necrotic, highly attenuated, and with dense cytoplasm, indicative of extreme fluid loss associated with necrosis. Patent pathways through the endothelial layer were seen associated with fluid accumulation in the subendothelial spaces. Such pathways are never seen in control endothelium and represent a very high conductance path for both plasma water and protein. The results are consistent with the hypothesis that epsilon-toxin interacts with endothelial cells of rat venular microvessels and increases the vessel wall permeability by direct damage of the endothelium. The answer to the broad question of whether the rat mesentery model might be useful to study endothelial interaction with blood-borne toxins such as epsilon-toxin is clear. The single perfused microvessel model does enable investigation of relatively small quantities of toxin with mammalian microvessel endothelium.

While this study is the first to measure biophysical permeability coefficients in response to epsilon-toxin, there are numerous previous reports indicating fluid and protein extravasation resulting from epsilon-toxin toxemia. Edema and excess protein extravasation in various tissues have been noted at necropsy in experimentally and naturally intoxicated animals (12, 14, 30, 38, 39, 43). Morphological and histochemical methods provide endpoint observations but do not enable finer

FIG. 6. TEM images of vessels perfused with control solutions illustrate endothelial appearance in the absence of epsilon-toxin exposure. (A) Luminal endothelial surface is smooth, and the nuclear chromatin (nuc) is moderately electron dense. (B) Detail of panel A shows endothelial cells are adherent to basement membrane (bm), and a pericyte (peri) with moderate electron density is seen within multiple layers of basement membrane closely associated with the endothelial cell. pv, plasmalemmal vesicle. (C) One further image shows the fine structure of an endothelial cell, including an intact junctional complex (jc), numerous vesicles, and a nucleus. The process of a fibroblast (fib) is present in the interstitial matrix.

temporal characterization of the progress of intoxication. Moreover, because the toxin has been shown to stimulate vascular smooth muscle contraction and elevate arterial pressure, it is very difficult to separate changes in vessel wall permeability, tissue perfusion, and intravascular pressure in whole-animal models (28, 36). Under the conditions of our experiments, the intravascular hydraulic pressure and protein osmotic pressure are directly controlled, while extravascular hydraulic pressure and protein osmotic pressure are negligible (2, 20). The postcapillary microvessels used in this study are not invested with smooth muscle cells and maintain a constant diameter under the conditions of these experiments. Individual measurements in the single perfused vessel, taking between 5 and 8 seconds, enable characterization of permeability changes

with temporal resolution comparable to cell culture methods used to measure transepithelial resistance, small solute flux, or hydraulic conductivity (18, 34, 41).

The single vessel technique was used here to measure an increase in water flux crossing the microvessel wall resulting from epsilon-toxin exposure and was also used to infer loss of the barrier to serum albumin (decrease of effective protein osmotic pressure shown in Fig. 1). Such changes in each of these parameters are directly important in the development of edema. However, one cannot specify what level of L_p measured in a single vessel would be correlated with organ failure or death. We can say that the ultrastructural examination of all vessels perfused with epsilon-toxin revealed endothelial cells that were severely damaged and regions of basement membrane exposed directly to the perfusate. These endothelial gaps were not found in control vessels. The latter control data agree with results of a much larger examination of normal endothelium in a recent publication from our group in which nearly 700 micrographs were analyzed for tight junction structure using rat mesentery microvessels that were perfused for 15 to 30 min with vehicle solutions similar to that in the present study (2). We also noted that once the L_p began to increase, removing perfusate containing toxin and reverting to control perfusate failed to return the L_p toward control. Indeed, in many instances when the perfusing pipette was removed at the end of an experiment and blood was allowed to return to the vessel, we observed hemostasis in vessels that had been perfused with toxin; vessels not perfused with toxin almost always resumed normal blood flow.

We also note that the effects of epsilon-toxin were not similar to an acute inflammatory response, such as that stimulated by platelet activating factor or other acute inflammatory mediators. The typical response of mesenteric postcapillary venules to platelet activating factor is an increase to a peak value within 10 to 15 min followed by a recovery phase lasting another 10 to 60 min (3). Similar patterns have been described for the inflammatory mediators histamine (21) and bradykinin (15) in these vessels. Because the epsilon-toxin effect exhibited a continuous L_p increase and did not return toward control, it did not have the characteristic pattern associated with an acute inflammatory response.

Previous studies of epsilon-toxin using MDCK cells have examined parameters different from the water flux and loss of protein osmotic pressure measured in the present study. Loss of intracellular K^+ (29, 33, 35), decreased cell viability (27, 29, 33–35), increased entry of propidium iodide (34, 35), and a fall in *trans*-epithelial resistance (34) all have been used to infer epsilon-toxin toxicity toward MDCK cell monolayers. None of these measures are directly comparable to increased L_p in microvessels. However, all these measures are indicative of damaged cell membrane and were correlated with compromised epithelial monolayer barrier function. The MDCK cell model is particularly sensitive to epsilon-toxin, and the concentrations of epsilon-toxin that elicited effects in the cited MDCK studies ranged from near 10^{-10} M to 10^{-8} M. In the *in vivo* mesentery microvessel, we found a threshold effect at $1 \mu\text{g ml}^{-1}$ (approximately 3×10^{-8} M) when measuring increases in L_p . Endothelial gaps seen with TEM and correlated with increased L_p could have formed as endothelial tight and adherens junctions released or could have resulted from cell

disruption. Some clostridial toxins inactivate small GTPases, leading to the breakdown of adherens and tight junctions in both epithelial and endothelial cells (1, 5, 42). In a recent study using MDCK monolayers, at a time and dose of epsilon-toxin that elicited a complete loss of *trans*-epithelial resistance, the monolayer permeability to a solute (dextran 38500) restricted to the paracellular pathway was not increased and the immunofluorescent distribution of peripheral band actin and components of the tight junction complex was not different from control (34). These data indicated that direct cell disruption by pore formation on the apical membrane preceded any possible direct effect on the junctional complex. Thus, it is likely that the broad areas of basement membrane exposed during epsilon-toxin treatment of the mesentery microvessels result from the disruption of the endothelial cells rather than from disassembly of cell-cell junctions.

The present results using *in vivo* endothelium and epsilon-toxin concentrations up to $10 \mu\text{g ml}^{-1}$ contrast with those of our previous study, in which the toxin at up to $120 \mu\text{g ml}^{-1}$ did not induce morphological changes in cultured aortic endothelial cells (tested from cattle, goats, and sheep) (40). One possibility for this difference was that activity of the toxin toward endothelium was dependent on a factor in blood not present in the culture medium. In our previous study we addressed that possibility by testing for toxicity against the endothelial cells in the presence as well as the absence of serum proteins, but we found that in either case there was no morphological change induced by the toxin. While most of the present experiments were carried out in physiological saline (Ringer's) solution supplemented with bovine serum albumin, in several experiments in which toxin was diluted into Ringer's solution without BSA, epsilon-toxin also induced large increases in L_p (not shown). Therefore, our results indicate that no other component of circulating blood is required for toxicity against the mesenteric venular microvessel endothelium.

A specific receptor for epsilon-toxin has not been identified. However, the presence of a receptor, particularly in brain and kidney, has been suggested by uptake of labeled toxin (6, 30). Also, *in vitro* studies showed that treatment of rat brain homogenates with neuraminidase or lipase reduced binding of epsilon-toxin, indicating that a receptor could be a sialoglycoprotein within a specific lipid environment (31). Following these earlier studies, recent work demonstrated that heptamers of epsilon-toxin were found exclusively associated with the detergent-resistant membrane fraction from MDCK cells, further demonstrating that epsilon-toxin binding is dependent on specific cholesterol-containing microdomains, possibly lipid rafts or caveolae (27). Therefore, it is possible that the aortic endothelial cells which showed no morphological response to epsilon-toxin did not express the appropriate receptor or associated microdomain. We cannot say at present whether those cells express such a domain *in vivo* or could do so under different culture conditions. The demonstration in the present experiments of the sensitivity of postcapillary microvessel endothelium to epsilon-toxin suggests that cultured endothelial monolayers from other sources or under different culture conditions should be tested and that an appropriate endothelial culture model could be found.

In our experiments we also noted damage to pericytes of the toxin-perfused microvessels. While some studies have sug-

gested that pericytes promote vascular permeability through contractility, recent studies have focused on the role of pericytes in the maturation of microvessels (17). Loss of pericytes, in addition to endothelial cells, due to epsilon-toxin could potentially exacerbate local permeability increases and delay return to normal vascular function in cases of sublethal intoxication.

The present study uses the mesenteric microvessel as a model for the direct vascular effects of epsilon-toxin. However, the principal lethal pathology in sheep is associated with brain and lungs. The traditionally accepted model to explain brain lesions due to epsilon-toxin (6) suggests that such lesions are due to the action of this toxin on endothelial cells, which generates perivascular edema followed by hypoxia and necrosis of the perivascular tissues. However, a recent study showed that epsilon-toxin can also leak into the brain and act directly on rat neurons, producing degeneration and necrosis of these cells in cases of subacute or chronic epsilon-toxin intoxication (11). The results of the present experiments confirm that direct damage does occur in endothelial cells exposed to epsilon-toxin. However, it is possible that epsilon-toxin acts both on endothelial cells and neurons alike. Moreover, it is possible and as yet unknown whether differences exist between various vascular beds in regard to toxin sensitivity and pathogenesis.

Summary. We have shown that epsilon-toxin, in a time- and dose-dependent manner, rapidly and irreversibly compromises the barrier function of venular microvessel endothelium in the rat mesentery. The toxin appeared to directly damage vascular endothelium, inducing formation of gaps in the endothelial cell layer correlated with loss of effective protein osmotic pressure and large increases in measured L_p . These changes are consistent with those expected to lead to rapid edema formation and would also promote access of the toxin to cells in the surrounding tissue. The single perfused vessel model will enable further investigation of epsilon-toxin interaction with the vascular endothelium in situ.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants from the National Heart, Lung, and Blood Institute (HL28607 and HL44485) and National Institute of Allergy and Infectious Diseases (AI56177).

We gratefully acknowledge the expert technical work of Joyce Lenz.

REFERENCES

- Adamson, R. H., F. E. Curry, G. Adamson, B. Liu, Y. Jiang, K. Aktories, H. Barth, A. Daigeler, N. Golenhofen, W. Ness, and D. Drenckhahn. 2002. Rho and rho kinase modulation of barrier properties: cultured endothelial cells and intact microvessels of rats and mice. *J. Physiol.* **539**:295–308.
- Adamson, R. H., J. F. Lenz, X. Zhang, G. N. Adamson, S. Weinbaum, and F. E. Curry. 2004. Oncotic pressures opposing filtration across non-fenestrated rat microvessels. *J. Physiol.* **557**:889–907.
- Adamson, R. H., M. Zeng, G. N. Adamson, J. F. Lenz, and F. E. Curry. 2003. PAF- and bradykinin-induced hyperpermeability of rat venules is independent of actin-myosin contraction. *Am. J. Physiol. Heart Circ. Physiol.* **285**:H406–H417.
- Beal, D. R., R. W. Titball, and C. D. Lindsay. 2003. The development of tolerance to *Clostridium perfringens* type D epsilon-toxin in MDCK and G-402 cells. *Hum. Exp. Toxicol.* **22**:593–605.
- Busch, C., and K. Aktories. 2000. Microbial toxins and the glycosylation of Rho family GTPases. *Curr. Opin. Struct. Biol.* **10**:528–535.
- Buxton, D., K. A. Linklater, and D. A. Dyson. 1978. Pulpy kidney disease and its diagnosis by histological examination. *Vet. Rec.* **102**:241.
- Buxton, D., and K. T. Morgan. 1976. Studies of lesions produced in the brains of colostrum deprived lambs by *Clostridium welchii* (Cl. perfringens) type D toxin. *J. Comp. Pathol.* **86**:435–447.
- Cole, A. R., M. Gibert, M. Popoff, D. S. Moss, R. W. Titball, and A. K. Basak. 2004. *Clostridium perfringens* epsilon-toxin shows structural similarity to the pore-forming toxin aerolysin. *Nat. Struct. Mol. Biol.* **11**:797–798.
- de Maagd, R. A., A. Bravo, C. Berry, N. Crickmore, and H. E. Schnepf. 2003. Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. *Annu. Rev. Genet.* **37**:409–433.
- Finnie, J. W. 1984. Ultrastructural changes in the brain of mice given *Clostridium perfringens* type D epsilon toxin. *J. Comp. Pathol.* **94**:445–452.
- Finnie, J. W., P. C. Blumberg, and J. Manavis. 1999. Neuronal damage produced in rat brains by *Clostridium perfringens* type D epsilon toxin. *J. Comp. Pathol.* **120**:415–420.
- Finnie, J. W., and P. Hajduk. 1992. An immunohistochemical study of plasma albumin extravasation in the brain of mice after the administration of *Clostridium perfringens* type D epsilon toxin. *Aust. Vet. J.* **69**:261–262.
- Gardner, D. E. 1973. Pathology of *Clostridium welchii* type D enterotoxaemia. II. Structural and ultrastructural alterations in the tissues of lambs and mice. *J. Comp. Pathol.* **83**:509–524.
- Griner, L. A., and W. D. Carlson. 1961. Enterotoxaemia of sheep. II. Distribution of I-131 radioiodinated serum albumin in brains of *Clostridium perfringens* type D intoxicated lambs. *Am. J. Vet. Res.* **22**:443–446.
- He, P., M. Zeng, and F. E. Curry. 1998. cGMP modulates basal and activated microvessel permeability independently of $[Ca^{2+}]_i$. *Am. J. Physiol.* **274**:H1865–H1874.
- He, P., X. Zhang, and F. E. Curry. 1996. Ca^{2+} entry through conductive pathway modulates receptor-mediated increase in microvessel permeability. *Am. J. Physiol.* **271**:H2377–H2387.
- Hellstrom, M., H. Gerhardt, M. Kalen, X. Li, U. Eriksson, H. Wolburg, and C. Betsholtz. 2001. Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. *J. Cell Biol.* **153**:543–553.
- Hippenstiel, S., S. Tannert-Otto, N. Vollrath, M. Krüll, I. Just, K. Aktories, C. von Eichel-Streiber, and N. Suttrop. 1997. Glucosylation of small GTP-binding Rho proteins disrupts endothelial barrier function. *Am. J. Physiol.* **272**:L38–L43.
- Huxley, V. H., F. E. Curry, and R. H. Adamson. 1987. Quantitative fluorescence microscopy on single capillaries: alpha-lactalbumin transport. *Am. J. Physiol.* **252**:H188–H197.
- Kajimura, M., H. Wiig, R. K. Reed, and C. C. Michel. 2001. Interstitial fluid pressure surrounding rat mesenteric venules during changes in fluid filtration. *Exp. Physiol.* **86**:33–38.
- Kendall, S., and C. C. Michel. 1995. The measurement of permeability in single rat venules using the red cell microperfusion technique. *Exp. Physiol.* **80**:359–372.
- Liu, J. W., A. G. Porter, B. Y. Wee, and T. Thanabalu. 1996. New gene from nine *Bacillus sphaericus* strains encoding highly conserved 35.8-kilodalton mosquitocidal toxins. *Appl. Environ. Microbiol.* **62**:2174–2176.
- McDonald, J. N., and J. R. Levick. 1993. Effect of extravascular plasma protein on pressure-flow relations across synovium in anaesthetized rabbits. *J. Physiol.* **465**:539–559.
- Michel, C. C. 1980. Filtration coefficients and osmotic reflexion coefficients of the walls of single frog mesenteric capillaries. *J. Physiol.* **309**:341–355.
- Michel, C. C., and F. E. Curry. 1999. Microvascular permeability. *Physiol. Rev.* **79**:703–761.
- Miyata, S., O. Matsushita, J. Minami, S. Katayama, S. Shimamoto, and A. Okabe. 2001. Cleavage of a C-terminal peptide is essential for heptamerization of *Clostridium perfringens* epsilon-toxin in the synaptosomal membrane. *J. Biol. Chem.* **276**:13778–13783.
- Miyata, S., J. Minami, E. Tamai, O. Matsushita, S. Shimamoto, and A. Okabe. 2002. *Clostridium perfringens* epsilon-toxin forms a heptameric pore within the detergent-insoluble microdomains of Madin-Darby canine kidney cells and rat synaptosomes. *J. Biol. Chem.* **277**:39463–39468.
- Nagahama, M., H. Iida, and J. Sakurai. 1993. Effect of *Clostridium perfringens* epsilon toxin on rat isolated aorta. *Microbiol. Immunol.* **37**:447–450.
- Nagahama, M., S. Ochi, and J. Sakurai. 1998. Assembly of *Clostridium perfringens* epsilon-toxin on MDCK cell membrane. *J. Nat. Toxins* **7**:291–302.
- Nagahama, M., and J. Sakurai. 1991. Distribution of labeled *Clostridium perfringens* epsilon toxin in mice. *Toxicon* **29**:211–217.
- Nagahama, M., and J. Sakurai. 1992. High-affinity binding of *Clostridium perfringens* epsilon-toxin to rat brain. *Infect. Immun.* **60**:1237–1240.
- Payne, D. W., E. D. Williamson, H. Havard, N. Modi, and J. Brown. 1994. Evaluation of a new cytotoxicity assay for *Clostridium perfringens* type D epsilon toxin. *FEMS Microbiol. Lett.* **116**:161–167.
- Petit, L., M. Gibert, D. Gillet, C. Laurent-Winter, P. Boquet, and M. R. Popoff. 1997. *Clostridium perfringens* epsilon-toxin acts on MDCK cells by forming a large membrane complex. *J. Bacteriol.* **179**:6480–6487.
- Petit, L., M. Gibert, A. Gouch, M. Bens, A. Vandewalle, and M. R. Popoff. 2003. *Clostridium perfringens* epsilon toxin rapidly decreases membrane barrier permeability of polarized MDCK cells. *Cell. Microbiol.* **5**:155–164.
- Petit, L., E. Maier, M. Gibert, M. R. Popoff, and R. Benz. 2001. *Clostridium perfringens* epsilon toxin induces a rapid change of cell membrane permeability to ions and forms channels in artificial lipid bilayers. *J. Biol. Chem.* **276**:15736–15740.
- Sakurai, J., M. Nagahama, and Y. Fujii. 1983. Effect of *Clostridium perfringens* epsilon-toxin on the cardiovascular system of rats. *Infect. Immun.* **42**:1183–1186.
- Thanabalu, T., and A. G. Porter. 1996. A *Bacillus sphaericus* gene encoding a novel type of mosquitocidal toxin of 31.8 kDa. *Gene* **170**:85–89.

38. **Uzal, F. A., and W. R. Kelly.** 1997. Effects of the intravenous administration of *Clostridium perfringens* type D epsilon toxin on young goats and lambs. *J. Comp. Pathol.* **116**:63–71.
39. **Uzal, F. A., W. R. Kelly, W. E. Morris, and R. A. Assis.** 2002. Effects of intravenous injection of *Clostridium perfringens* type D epsilon toxin in calves. *J. Comp. Pathol.* **126**:71–75.
40. **Uzal, F. A., B. E. Rolfe, N. J. Smith, A. C. Thomas, and W. R. Kelly.** 1999. Resistance of ovine, caprine and bovine endothelial cells to *Clostridium perfringens* type D epsilon toxin in vitro. *Vet. Res. Commun.* **23**:275–284.
41. **van Nieuw Amerongen, G. P., R. Draijer, M. A. Vermeer, and V. W. van Hinsbergh.** 1998. Transient and prolonged increase in endothelial permeability induced by histamine and thrombin: role of protein kinases, calcium, and RhoA. *Circ. Res.* **83**:1115–1123.
42. **Waschke, J., W. Baumgartner, R. H. Adamson, M. Zeng, K. Aktories, H. Barth, C. Wilde, F. E. Curry, and D. Drenckhahn.** 2004. Requirement of Rac activity for maintenance of capillary endothelial barrier properties. *Am. J. Physiol. Heart Circ. Physiol.* **286**:H394–H401.
43. **Worthington, R. W., and M. S. Mulders.** 1975. Effect of *Clostridium perfringens* epsilon toxin on the blood brain barrier of mice. *Onderstepoort J. Vet. Res.* **42**:25–27.

Editor: D. L. Burns