Ultrastructural changes of lung capillary endothelium in response to botulinum C2 toxin

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Ultrastructural changes of lung capillary endothelium in response to botulinum C2 toxin. J. Appl. Physiol. 82(2): 382-388, 1997.—The role of the endothelial cytoskeleton for the structural integrity of the pulmonary gas exchange area was probed with the use of Clostridium botulinum C2 toxin. This agent causes selective loss of nonmuscle F-actin. In buffer-perfused rabbit lungs, vascular pressures were kept within physiological ranges. In different groups, low-dose [0.3 (C2,I)/0.6 (C2,II) ng/ml] and high-dose [10 (C2,I)/20 (C2,II) ng/ml] toxin were applied into the buffer fluid; experiments were terminated after a total weight gain of either 1 or 7.5 g. Electron microscopy revealed extensive attenuations, undulations, and protrusions of the endothelial layer, suggestive of “remodeling” and “flowing” of the cell membrane in low C2 toxin-treated lungs accompanied by few disruptions of the endothelial layer and edema formation. In addition, endothelial cells displayed vesiculation and bleb formation. Lungs that were exposed to high-toxin doses displayed marked attenuations of the endothelial layer in addition to large endothelial cell disruptions, which did not include interendothelial junctions. Interestingly, type II epithelial cells displayed fusion of lamellar bodies. Collectively, these data suggest that the actin microfilament system is instrumental in supporting endothelialcellmembraneconfigurationandintegrityandmaintainsinthintinalbarrierfunctionofthelungmicrovasculature.

bacterial exotoxins; lung edema; pulmonary endothelial cells; rabbit

PULMONARY EDEMA was commonly divided into two models thought to be caused by basically different underlying events. One is hydrostatic pulmonary edema, in which an elevated lung capillary pressure forces transudation of fluid into the interstitium and, if severe enough, into the alveolar spaces. The other is high permeability pulmonary edema, characterized by increased fluid and protein leakage due to inflammatory or toxic injury to the endothelial and possibly epithelial layer (7, 29). However, there has been increasing evidence that the two models may be two steps of the same phenomenon (2, 3, 7, 36). In previous ultrastructural studies in rabbit lungs, we focused on the lung vascular leakage evoked by the bacterial exotoxins Staphylococcus aureus α-toxin and Escherichia coli hemolysin. The pore-forming staphylococcal agent was noted to cause severe alterations of the capillary endothelial cells, including an increased electron density of the cell nuclei and detachment of the cells from the basement membrane; moreover, subsequent wide-spread interstitial edema was obvious (27). In contrast, although effecting comparable amounts of interstitial fluid accumulation, the permeabilizing effect of E. coli hemolysin occurred in the absence of visible damage to endothelial or epithelial cells and in the absence of cell detachment (13).

Recent studies on the ultrastructural damage of the gas exchange area in rabbit lungs after exposure to high capillary pressures revealed a further distinct pattern of barrier lesions. Disruptions of both endothelial and epithelial layers in variable association with additional rupture of the basement membrane were visualized (2, 3, 6, 10, 31, 37). Interestingly, almost no breaks occurred at intercellular junctions, the mechanical strength of which thus appears to be higher than that of the cell membranes themselves. The barrier lesions that occurred under conditions of high capillary pressure were attributed to stress failure; the mechanisms that maintained cellular integrity and anchorage of cell-to-cell and cell-to-matrix contacts are overridden by the mechanical forces. Major importance for the maintenance of the endothelial and epithelial barrier function is attributed to the cytoskeleton. Actin filament stress fibers in endothelial cells may increase the endothelial resistance to hemodynamic shear forces (9, 15, 38). The cytoskeletal fiber meshwork serves to anchor intercellular junctions (17, 25, 26) and structurally supports the cell membrane as a plasmalemmal undercoat (18).

In a recent study, Ermert et al. (11) used Clostridium botulinum C2 toxin as a tool for selective destruction of nonmuscle F-actin in perfused rabbit lungs and demonstrated a dose-dependent dramatic increase in the capillary filtration coefficient. Maneuvers with elevation of the lung capillary pressure (step increase by 7.5 mmHg) provoked extensive interstitial fluid leakage, and electron-microscopic examination of high-dosage toxin-challenged lungs revealed attenuations and disruptions of endothelial cells. In the present study, we extend these morphological studies by following a protocol that avoided any hydrostatic challenge and compared a high and a low C2 toxin dose. Progressive edema formation was again provoked, in conjunction with dramatic ultrastructural changes of the lung capillary endothelial cells: attenuations and disruptions in response to high-dose toxin exposure and protrusions of the thinned endothelial membrane, accompanied by marked vesiculation and bleb formation, in response to low-dose toxin stimulation. These alterations strongly suggest a basic role of the actin microfilament system, known to be part of the plasmalemmal...
undercoat, in maintaining the structure and integrity of the lung capillary endothelial cell membranes under conditions of physiological intravascular pressures.

MATERIALS AND METHODS

Isolated Lung Model

The isolated lung model has previously been described (28). Briefly, rabbits of either gender (2.2–2.6 kg body wt) were deeply anesthetized and anticoagulated with 1,000 U heparin/kg body wt. The lungs were excised while they were perfused with Krebs-Henseleit buffer through cannulas in the pulmonary artery and the left atrium. The buffer contained 132.8 mM NaCl, 4.3 mM KCl, 1.1 mM KH₂PO₄, 24.1 mM NaHCO₃, 2.4 mM CaCl₂, and 1.3 mM MgPO₄ as well as 240 mg glucose and 5 g hydroxyethylamylopectine as oncotic agent per 100 ml. The lungs were placed in a temperature-equilibrated housing chamber at 37°C, freely suspended from a force transducer. They were ventilated with 4% CO₂-17% O₂-79% N₂ (tidal volume 30 ml; frequency 30/min; end-expiratory pressure 1 cmH₂O), and the pH of the perfusion fluid ranged between 7.35 and 7.45. After extensive rinsing of the vascular bed, the lungs were perfused in a recirculating system with a pulsatile flow of 150 ml/min. The alternate use of two separate perfusion circuits, each containing 200 ml, allowed exchange of perfusion fluid. Perfusion pressure, ventilation pressure, and the weight of the isolated organ were registered continuously. The left atrial pressure was set to 2 mmHg under baseline conditions (0 referenced at the hilum). Lungs selected for the study were those that 1) had a homogeneous white appearance without signs of hemostasis or edema formation, 2) had pulmonary artery and ventilation pressures in the normal range, and 3) were isogravimetric during an initial steady-state period of 40 min.

Preparation of C₂ Toxin

Preparation of components I (C₂₁) and II (C₂₁I) of the toxin was performed essentially as described (24). Aliquots of one batch of each toxin component, dissolved in saline, were stored at −20°C and used throughout the study. Mixture of the components was performed directly before experimental use.

Experimental Design

Four experimental groups were investigated (n = 3 lungs each).

Control lungs. Control lungs were perfused in the absence of C₂ toxin. To match the longest perfusion times of the other experimental groups (see below), extracorporeal circulation was continued for 180 min.

Low-dose C₂ toxin. Admixture of C₂ toxin to the perfusion fluid in a concentration of 0.3 (C₂₁)/0.6 (C₂₁I) ng/ml. Experiments were terminated when significant edema formation occurred (7.5 g weight gain).

High-dose C₂ toxin. Admixture of C₂ toxin to the perfusion fluid in a concentration of 10 (C₂₁)/20 (C₂₁I) ng/ml. Experiments were terminated at the onset of edema formation (1 g weight gain).

High-dose C₂ toxin. Admixture of C₂ toxin to the perfusion fluid in a concentration of 10 (C₂₁)/20 (C₂₁I) ng/ml. Experiments were terminated when significant edema formation had occurred (7.5 g weight gain).

Tissue Preparation for Electron Microscopy

Immediately after termination of perfusion at the preset levels of weight gain (preset time in control lungs), lungs were inflated to two-thirds of their total lung capacity. They were fixed by perfusion with potassium phosphate-buffered 2.5%
glutaraldehyde solution (pH 7.4, effective osmolarity 340 mosM) at a hydrostatic pressure of 20 cmH2O for 20 min. Subsequently, the entire lung was immersed in the same glutaraldehyde solution overnight. Six samples from each lung were randomly selected from the upper, middle, and lower segments of the central and peripheral lung regions. The samples were postfixed with 1% osmium tetroxide, dehydrated in a graded series of alcohol, and embedded in Spurr’s medium. Ultrathin sections of 70–80 nm were cut, placed on 200-mesh-copper grids, and contrasted with uranyl acetate and lead citrate. Microscopy was performed with a model CEM 902 Zeiss transmission electron microscope.

RESULTS

Because the objective of this study was to avoid any hydrostatic pressure elevation, the pulmonary artery pressure ranged between 5 and 8 mmHg throughout all lung experiments. Any spontaneous weight gain did not occur.

Control Lungs

Control lungs displayed no weight gain over the entire perfusion period of 180 min. Normal lung architecture was maintained throughout as previously shown (27). In particular, there was no evidence of interstitial or alveolar edema formation, and signs of cellular degeneration were missing. Due to the use of blood-free perfusate, no erythrocytes or thrombocytes were seen in the microvasculature of these lungs, whereas granulocytes, lymphocytes, and monocytes were noted to remain adherent to the lung capillary endothelial cells even under these conditions (12).

Exposure to Low-Dose C2 Toxin

Admixture of C2 toxin to the perfusion fluid in a concentration of 0.3 (C2, I) / 0.6 (C2, II) ng/ml caused the preset level of 7.5 g weight gain after a total perfusion period of 122 ± 16 (SD) min. Interstitial fluid accumulation was observed in these lungs (Figs. 1–3), mainly restricted to the thick side of the alveolar septum. Impressive ultrastructural changes of the capillary intimal surface were noted, mainly characterized by elongation, folding, vesiculation, and bleb formation of the endothelial layer. Few endothelial disruptions were noted in this group. In contrast, no visible changes of the endothelial cell nucleus and the interendothelial junctions were detected. The marked foldings and undulations of the endothelial layer were accompanied by some moderate irregularities and foldings of the epithelial layer. Marked fusion of the lamellar bodies within the type II epithelial cells was noted, and increased numbers of lamellar bodies fused with the cell membrane, indicating enhanced secretion.

Exposure to High-Dose C2 Toxin

Admixture of C2 toxin to the perfusion fluid in a concentration of 10 (C2, I) / 20 (C2, II) ng/ml caused onset of edema formation after a total perfusion period of 16 ± 3 min (group C). In these lungs, no significant interstitial fluid accumulation was visualized. Ultrastructural
changes were mainly restricted to the endothelial layer. A few endothelial cells displayed attenuations, which were located both at the thin side of the capillary wall, fused by its basal lamina with the basal lamina of type I epithelial cells, and at the thick side of the capillary wall, facing the connective tissue sheet of the interalveolar septum. There were no ultrastructural changes of the endothelial cell nucleus and the interendothelial junctions.

Prolonged recirculation of 10 (C2,1)/20 (C2,II) ng/ml C2 toxin caused a total lung weight gain of 7.5 g within 38 ± 6 min (group D). In these lungs, interstitial fluid accumulation was evident (Fig. 4), preferentially distributed to the thick side of the capillary wall. At the thin side of the capillary walls, edema was limited to small sites with disruption of the basal lamina. Attenuations of endothelial cells were even more impressive than in the lungs exposed for short terms to the high toxin dose; its localization again included both the thin and thick sides of the capillary wall. Moreover, marked disruption of the endothelial layer became visible. Such interruptions were often encountered in the vicinity of the interendothelial junctions. No ultrastructural changes of type I epithelial cells and interepithelial junctions were seen in the lungs of groups C and D. Similarly, as described for the low-dose toxin exposure, the recirculation of 10 (C2,1)/20 (C2,II) ng/ml C2 toxin provoked fusion of the epithelial type II cell lamellar bodies accompanied by fusion of these bodies with the cell membrane.

**DISCUSSION**

The present study employed the model of perfused rabbit lungs to investigate the role of the actin microfilament system in maintaining the integrity of the capillary-endothelial and alveolo-epithelial barriers. As previously described by our laboratory, normal lung architecture was observed even after a total period of extracorporeal buffer perfusion of >3 h (12, 27). In these preceding studies, morphometry of endothelium-adherent blood cells was performed; and substantial numbers of granulocytes, lymphocytes, and monocytes were noted to remain resident in the lung microvasculature during ex vivo buffer perfusion (12, 14). In contrast, erythrocytes and platelets were completely washed out under these conditions. These findings, although not quantified by morphometry, were corroborated in the present investigation.

To probe the role of the actin-based cytoskeleton in the maintenance of the barrier functions of the lung gas-exchange area, we employed the botulinum C2 toxin to induce selective loss of nonmuscle F-actin. This binary toxin acts on monomeric G-actin via ADP-ribosylation (1, 35), which blocks its contribution to polymerization. Moreover, ADP-ribosylated G-actin functions
as a capping protein, effecting progressive decay of F-actin and impairing the ability of the cell to maintain and remodel its microfilament system. Botulinum C2 toxin is thus much more specific than the cytochalasins previously used for probing the role of the cytoskeleton. These agents may promote cell activation themselves (30); and different cellular target sites for the cytochalasins have been identified, resulting in a complex interaction with the microfilamentous network (32, 34).

As observed in Ermert et al. (11), high-dose C2 toxin challenge resulted in marked attenuations and disruptions of the endothelial layer. Notably, according to the protocol of the present investigation, all hydrostatic challenges were avoided, the total amount of edema accumulation was restricted to 1 g (group C) and 7.5 g (group D), and the lung vascular pressure was documented to range within physiological range throughout the experiments. Any “mechanical” cause of the striking ultrastructural changes of the endothelial cells can thus be excluded, in contrast to the disturbances reported as capillary “stress failure” (6, 10, 31, 37). The ultrastructural changes observed in response to C2 toxin clearly differ from those evoked by staphylococcal α-toxin (27) or E. coli hemolysin (13). Thus presenting as a distinct entity. The gaps within the endothelial layer were interpreted to be endothelial cell disruptions rather than interendothelial separation phenomena. Considering the basic mechanism of injury of the C2 toxin on the actin-based microfilament system, the marked attenuations and disruptions of the capillary endothelial cells in response to high toxin challenge may be related to effects of this agent on the cytoskeleton-based plasmalemmal undercoat. This has been described to function as a membrane “spacer,” particularly in areas of cell attenuation (18, 21). Disruption of the plasmalemmal undercoat may then result in a loss of structural support of the cell membrane configuration, with subsequent phenomena of membrane fusion, gap formation, and interstitial edema in the vicinity of these lesions.

This view is substantially supported by the studies with long-term exposure of the isolated rabbit lungs to low doses of C2 toxin. The predominant lesions under these conditions were marked attenuation, elongation, undulation, and protrusions of the endothelial layer. Our hypothesis is that such alterations may represent extensive “remodeling” and “flowing” of the cell membranes after loss of structural support by the actin-based plasmalemmal undercoat, which of course cannot be fully proved by this study. The fact that these phenomena of membrane remodeling are much more impressive than overt disruptions of the endothelial layer (in contrast to the lungs of groups C and D) may be explained by the long-term exposure of the lungs to low doses of the toxin.

In both low- and high-dose toxin-exposed lungs, interendothelial junctions were never found to be involved in the process of gap formation. In the lungs of groups C and D, however, disruptions of the cell membrane were often noted to occur in the vicinity of these cell-to-cell adhesion structures. Such a gap formation has similarly been described for lungs exposed to supranormal transmural capillary pressures (6, 36). These findings may thus suggest that the cellular anchorage of the intercellular junctions may represent a particularly stable region (16), not as readily affected by C2 toxin or mechanical stress as other membrane domains. The mechanical strength and rigidity of the intercellular junctions may then render the cell membranes in the vicinity of these structures more vulnerable to mechanical failure (6).

In lungs treated for long terms with the low dose of C2 toxin (group B), increased numbers of plasmalemmal vesicles were noted in the endothelial cell layer, accompanied by impressive bleb formation in some areas. Such vesiculation is reminiscent of findings in rabbit lungs, in which prolonged edema formation was provoked by exposure to low doses of E. coli hemolysin (13). As discussed in the latter report (13), these findings may reflect enhanced secretory activity of the endothelial cell type or, more likely, active transcellular fluid transport, suggested as a defense mechanism against edema formation (4, 5, 8). The rapidity of the edema accumulation and/or the severity of endothelial lesions in the lungs exposed to the high C2 toxin dose may explain why such endothelial vesicle formation was not evident in groups C and D.

In addition to the endothelial layer, the barrier characteristics of the alveolar epithelium might be affected by the C2 toxin treatment. The microfilament system is known to be involved in the maintenance of cell configuration and tight junctional competence of epithelial cell layers of different origin (19, 20, 33). The intestinal epithelium has been shown to become leaky in C2 toxin-poisoned mice (22, 23). The present electron-microscopic study did not, however, reveal any major alterations of the alveolar type I epithelial cells. In particular, attenuations, disruptions, and bleb formation, as observed in rabbit lungs exposed to high capillary transmural pressure, were not noted. It is conceivable that endothelial cells were preferentially injured due to intravascular application of the bacterial toxin, which may result in only minor concentrations of toxin reaching the type I pneumocytes. Alternatively, type I pneumocytes may be less sensitive to C2 toxin. This latter possibility seems more likely due to the observed effects of the toxin on type II pneumocytes, in which marked fusion and secretion of lamellar bodies were noted. Such alterations may be related to a loss of cytoskeletal control of compartmentalization. These findings are similar to recent in vitro findings in C2 toxin-exposed cultured type II pneumocytes (F. Griminger, unpublished observations), suggesting a significant distribution of the intravascularly applied toxin at least into this epithelial cell type under conditions of isolated lung perfusion. Further studies that use a transbronchial route for alveolar toxin deposition are required to settle the question of C2 toxin efficacy on alveolar epithelial barrier characteristics in greater detail.
Collectively, the present morphological study of C2 toxin-poisoned lungs strongly supports the notion that the actin microfilament system is operative in the maintenance of microvascular endothelial barrier function in intact organs with normal extracellular matrix and functional compartmentalization. It apparently serves to stabilize endothelial cell membrane configuration and integrity, dependent on the regimen of C2 toxin application, attenuations and disruptions of the endothelial layer as well as undulations and protrusions of its cell membranes are observed. Selective loss of actin appears to lead to an inability to resist the stress of transmural capillary pressures of physiological magnitude.

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