Requirement of Rac activity for maintenance of capillary endothelial barrier properties

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1 Institute of Anatomy and Cell Biology, University of Würzburg, D-97070 Würzburg; 2 Department of Pharmacology and Toxicology, Albert-Ludwigs-University, D-79104 Freiburg, Germany; and 3 Department of Human Physiology, University of California, Davis, California 95616

Submitted 13 March 2003; accepted in final form 18 September 2003

Waschke, J., W. Baumgartner, R. H. Adamson, M. Zeng, K. Aktories, H. Barth, C. Wilde, F. E. Curry, and D. Drenckhahn. Requirement of Rac activity for maintenance of capillary endothelial barrier properties. Am J Physiol Heart Circ Physiol 286: H394–H401, 2004.—Our previous experiments indicated that GTPases, other than RhoA, are important for the maintenance of endothelial barrier integrity in both intact microvessels of rats and mice and cultured mouse myocardial endothelial (MyEnd) cell monolayers (J Physiol 539: 295–308, 2002). In the present study, we inhibited the endothelial GTPase Rac by Clostridium sordellii lethal toxin (LT) and investigated the relation between the degree of inhibition of Rac by glucosylation and increased endothelial barrier permeability. In rat venular microvessels, LT (200 ng/ml) increased hydraulic conductivity from a control value of 2.5 ± 0.6 to 100.8 ± 18.7 × 10−7 cm s−1 cm H2O−1 after 80 min. In cultured MyEnd cells exposed to LT (200 ng/ml), up to 60% of cellular Rac was glucosylated after 90 min, resulting in depolymerization of F-actin and interruptions of junctional distribution of vascular endothelial cadherin (VE-cadherin) and β-catenin as well as the formation of intercellular gaps. To understand the mechanism by which inhibition of Rac caused disassembly of adherens junctions, we used laser tweezers to quantify VE-cadherin-mediated adhesion. LT and cytochalasin D, an actin depolymerizing agent, both reduced adhesion of VE-cadherin-coated microbeads to the endothelial cell surface, whereas the inhibitor of Rho kinase Y-27632 did not. Stabilization of actin filaments by jasplakinolide completely blocked the effect of cytochalasin D but not of LT on bead adhesion. We conclude that Rac regulates endothelial barrier properties in vivo and in vitro by 1) modulation of actin filament polymerization and 2) acting directly on the tether between VE-cadherin and the cytoskeleton, vascular endothelial cadherin; adherens junction; permeability; Rho proteins; actin

THE PRIMARY PHYSIOLOGICAL FUNCTION of the endothelium lining the inner surface of blood vessels is to create a selective barrier between blood and tissues. Barrier function is mainly regulated by opening and closure of gaps within the intercellular space that controls the passage of macromolecules and leukocytes across the vascular wall (21, 24). The width of the intercellular space is largely regulated by the endothelial actin cytoskeleton, which is attached to the inner surface of adherens-type intercellular junctions (9, 10). The predominant adhesion molecule of endothelial adherens junctions is vascular endothelial cadherin (VE-cadherin; cadherin 5), the cytoplasmic tail (cytointegrin) of which is linked to actin filaments via catenin-type adaptor molecules, α-, β-, and γ-catenin. Local breakdown of the endothelial barrier is a hallmark of the vascular response to a variety of permeability-increasing inflammatory agents (21). Accumulating evidence indicates that members of the Ras homology (Rho) family of small GTPases, Rho, Rac, and Cdc42, are important regulators of endothelial barrier properties by influencing both the endothelial actin-based cytoskeleton and the integrity of interendothelial junctions (23). Activated Rho acts primarily via Rho kinase (ROCK) to stimulate myosin-based contractility by upregulating phosphorylation of myosin light chain (2). The roles of Rac and Cdc42 in endothelial barrier regulation are less well understood.

Previous studies have demonstrated that inhibitory glucosylation of Rho family proteins by Clostridium difficile toxin B (assumed to inhibit Rho, Rac, and Cdc42) caused breakdown of the endothelial barrier in vitro (17) and in vivo (1). In contrast, selective inhibition of Rho activity by Clostridium botulinum C3 transferase-mediated ADP ribosylation or inactivation of ROCK by compound Y-27632 improved endothelial barrier properties both in vitro and in vivo (1, 2, 28). This indicates that toxin B-mediated breakdown of the endothelial barrier is due to inactivation of Rac or Cdc42 rather than to inactivation of Rho. Overexpression of active as well as constitutively active Rac by transfection of cultured endothelial cells with adenovirus expressing these proteins caused, within 14–16 h after transfection, a significant increase of transfer of FITC-dextran through monolayers grown on filters, further supporting the notion that normal Rac activity is important for endothelial barrier function (29). In contrast, transfection studies with constitutively active and inactive Cdc42 displayed no significant effect on monolayer permeability and integrity (26, 28, 29).

On the basis of these observations and our own experience with the influence of toxin B and Y-27632 on capillary permeability, the present study was undertaken to selectively inhibit Rac in capillary endothelial cells and to analyze the functional consequences of Rac inactivation on endothelial barrier properties in vitro and in vivo. In vivo experiments require rapid inactivation of Rac within a time window of 2 h. For this purpose, we used lethal toxin (LT) from Clostridium sordellii (8) at concentrations shown by parallel biochemical experiments to selectively inactivate Rac but not Rho and
Cdc42 within 60–120 min after application. Under these conditions, LT increased capillary hydraulic conductivity ($L_p$) in vivo and caused dissociation of endothelial junctions in vitro. Barrier breakdown was accompanied by simultaneous loss of F-actin.

To discriminate between the general (unspecific) consequences of F-actin breakdown on barrier integrity versus a direct modulatory role of Rac inhibition on VE-cadherin-mediated adhesion, we quantified VE-cadherin adhesion by a laser tweezers approach. This assay is based on microbeads coated with dimeric VE-cadherin ectodomains. The beads are allowed to settle on the surface of cultured microvascular endothelial cells, where the beads recruit endothelial VE-cadherin, β-catenin, and actin and induce assembly of typical adherens-type contacts at the free cellular surface (5). Adhesion of beads was probed by forces exerted by trapping the beads into the focus of a laser beam placed by microscopic control immediately above individual microbeads.

**MATERIALS AND METHODS**

**Cell culture.** The immortalized mouse microvascular endothelial (MyEnd) cell line was grown in DMEM (Life Technologies; Karlsruhe, Germany) supplemented with 50 U/ml penicillin G, 50 μg streptomycin, and 10% FCS (Biochrom; Berlin, Germany) in a humidified atmosphere (95% air-5% CO2) at 37°C. Culture conditions have been described before (1, 15). The cultures were allowed to settle on the surface of cultured microvascular endothelial cells, where the beads recruit endothelial VE-cadherin, β-catenin, and actin and induce assembly of typical adherens-type contacts at the free cellular surface (5). Adhesion of beads was probed by forces exerted by trapping the beads into the focus of a laser beam placed by microscopic control immediately above individual microbeads.

**Glucosylation assay.** MyEnd cells were incubated with various concentrations of LT in culture medium for various time intervals under cell culture conditions as described in Cell culture. Activity and specificity of toxin preparations were analyzed as described in Glucosylation assay (1, 19). The ROCK inhibitor Y-27632 (Calbiochem) at 10 μM was used at 10 μg/ml and cytochalasin D (Sigma; St. Louis, MO) at 10 μg/ml.

**Coating of polystyrene beads.** After being vortexed, a 10-μl solution of protein A-coated superparamagnetic polystyrene microbeads (Dynabeads, diameter 2.8 μm, Dynal; Oslo, Norway) containing 2 × 109 beads/ml was washed three times using 100 μl of buffer A (100 mM sodium phosphate buffer; pH 8.1). Washings were performed by immobilization of beads for 1 min in a magnetic tube holder (MPC-E-1, Dynal) and reuptake in the corresponding buffer. Washed beads were suspended in 100 μl of buffer A in HBSS containing 10 μg of either VE-cadherin-Fc or of the Fc part of human IgG (for control experiments) and allowed to react for 16 h at 4°C under permanent slow overhead rotation to avoid aggregation. After washing 3 × 5 min in 100 μl of buffer A and 3 × 5 min in buffer B (100 mM sodium borate; pH 9.0), beads were incubated for 45 min at RT in 100 μl of buffer B containing 0.54 mg dimethyl pimelimidate dihydrochloride (DMP; Pierce) to covalently cross-link protein A and bound Fc parts. After being washed 2 × 5 min in buffer C (100 μl of 0.2 M ethanolamine; pH 8.0), beads were incubated in buffer C for 2 h at RT. Finally, beads were washed 3 × 5 min in HBSS and stored in HBSS at 4°C for up to 8 days under permanent slow overhead rotation to avoid aggregation of beads. The concentration of beads in these stocks was ~1.6 × 106 beads/ml.

**Laser tweezers.** The home-built laser tweezers setup consisted of a Nd:Yag laser (1.064 μm), the beam of which was expanded to fill the back aperture a high-numerical aperture objective (100 × 1.3 oil, Zeiss; Oberkochen, Germany), coupled through the epi-illumination port of an Axiosvert 135 microscope (Zeiss) and reflected to the objective by a dichroic mirror (FT 510, Zeiss). Through all experiments, the laser intensity was 40 mW in the focal plane. Coated beads (10 μl of stock solution) were suspended in 500 μl of culture medium and allowed to interact with MyEnd monolayers for 30 min at 37°C.
before the initiation of experiments (addition of various compounds). Beads were considered tightly bound when resisting laser displacement at the 40-nM setting. For every condition, 100 beads were counted. The percentage of beads resisting laser displacement under various experimental conditions was normalized to control values.

Animal preparation. Rats were kept under conditions that conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and experiments were approved by the Institutional Animal Care and Use Committee of the University of California-Davis. Rats (male, Sprague-Dawley, 350–450 g, Hilltop Laboratory Animals) were anesthetized with pentobarbital sodium (65 mg/kg body wt) given subcutaneously. Anesthesia was maintained by giving additional pentobarbital (3 mg/dose) as needed. At the end of the procedure, rats were killed by pentobarbital overdose. We used male rats to avoid potential variability due to hormonal fluctuations in females.

Preparation of rats for \( L_p \) measurement. Anesthetized rats were placed on a heating pad to maintain normal body temperature. A midline surgical incision of \( \sim 1 \) cm was made in the abdominal wall, and the mesentery was gently taken out and spread over a pillar. The upper surface of the mesentery was continuously superfused with mammalian Ringer solution (temperature 37°C). All the experiments were carried out in straight nonbranched segments of venular microvessels, which were typically 25–35 \( \mu \)m in diameter. All vessels selected had brisk blood flow and were free of white blood cells.

Measurement of \( L_p \) of the microvessel wall. Measurements were based on the modified Landis technique, which measures the volume flux of water across the wall of a microvessel perfused via a glass micropipette after occlusion of the vessel. The assumptions and limitations of the measurement have been evaluated in detail (21). The volume flux per unit surface area of the vessel wall (\( J_S \)) was estimated during single occlusions, lasting \( \sim 10 \) s each, at constant hydraulic pressure (usually 50 cmH\(_2\)O) with the assumption that the net effective pressure determining fluid flow (\( P_{net} \)) was equal to the applied hydraulic pressure minus 3 cmH\(_2\)O, the approximate oncotic pressure contributed by BSA in all perfusates (10 mg/ml). \( L_p \) was estimated for each occlusion as (\( J_S / P_{net} \)). All perfusates were mammalian Ringer solution additionally containing serum albumin at 10 mg/ml (Sigma A 4378). \( L_p \) was added to the perfusate and delivered via the micropipette continuously. Measurements of initial (\( J_S \)) were made at \( \sim 10 \)-min intervals for up to 80 min in the presence and absence of LT. In preliminary experiments, \( L_p \) at 100 ng/ml was perfused through two venules for 150 min with no change from baseline \( L_p \). Therefore, we used 200 ng/ml LT in subsequent venule perfusion experiments.

Statistics. Values are expressed as means \( \pm \) SE. Statistical significance was assumed for \( P < 0.05 \). Because of the small sample size, we used nonparametric ANOVA (Kruskal-Wallis test) to examine the effects of LT on \( L_p \) over time.

RESULTS

LT increased permeability in rat microvessels in vivo. We perfused single microvessels of the rat mesentery to determine the role of Rac-mediated permeability regulation in vivo. Previously, we have shown that toxin B (100 ng/ml) increased permeability (i.e., \( L_p \)) within 120 min, whereas Y-27632 slightly reduced basal permeability (1). In five venules, LT (200 ng/ml) significantly increased microvessel \( L_p \) from a control value of 2.5 \( \pm \) 0.6 \( \times \) 10\(^{-7} \) to 80.6 \( \pm \) 19.2 \( \times \) 10\(^{-7} \) cm\(^{-1}\)cmH\(_2\)O\(^{-1}\) \( \times \) s\(^{-1} \) after 60 min and to 100.8 \( \pm \) 18.7 \( \times \) 10\(^{-7} \) cm\(^{-1}\)cmH\(_2\)O\(^{-1}\) \( \times \) s\(^{-1} \) after 80 min of treatment (Kruskal-Wallis test, \( P < 0.05 \)). Control perfusions in rat mesentery microvessels showed no change in \( L_p \) over 2 h (1). Figure 1 shows one experiment. Permeability began to rise after a lag phase of \( \sim 40 \) min. This time corresponds to the time in cultured MyEnd cells

where first changes in junctional integrity in VE-cadherin-mediated adhesion became detectable during LT treatment (see Fig. 3). These experiments indicate that Rac activity is important for maintenance of capillary barrier properties in vivo.

Glucosylation of Rho proteins by LT. To exclude the possibility that the LT preparation used in the present study might not only inactivate Rac but also Rho and Cdc42, we incubated recombinant RhoA, Rac-1, and Cdc42 with LT (200 ng/ml) for 120 min, as described in detail previously (19). Under these conditions, LT selectively glucosylated Rac but only weakly glycosylated Cdc42 and not RhoA (Fig. 2A). The degree of glucosylation was determined from the incorporation of \( [\text{14}C] \)p-glucose. After 120 min, Rac-1 was glucosylated 6- to 10-fold more than other small GTPases, RhoA or Cdc42 (Fig. 2A, inset). In contrast, toxin B (100 ng/ml, 150 min) induced strong glucosylation of all GTPases, RhoA, Rac-1, and Cdc42. We also measured glucosylation of Rac-1 in cultured MyEnd cells after varying times of exposure to 200 ng/ml LT. After 90 min, \( \sim 60\% \) of cellular Rac-1 was glucosylated and unavailable for \( [\text{14}C] \)glucose incorporation in cellular lysates (Fig. 2B). Prominent cellular changes were observed within 60–90 min of incubation with LT (see Fig. 3).

Effect of toxins on morphology and F-actin content of cultured endothelial cells. In untreated MyEnd monolayers, VE-cadherin and β-catenin displayed continuous immunolabeling of intercellular adherens junctions (Fig. 3, a and c). This continuous adherens belt displayed multiple interruptions in response to treatment with LT (200 ng/ml) for 60 min (Fig. 3, c and g). The first changes became visible at 40 min of treatment. Junctional sites lacking VE-cadherin and β-catenin immunoreactivity coincided with the appearance of intercellular gaps. Inhibition of ROCK with Y-27632 (30 μM, 90 min) neither caused interruptions of adherens belt nor the formation of intercellular gaps (Fig. 3, b and f). Cytochalasin D (10 μM, 60 min), an inhibitor of actin polymerization, caused interruptions of junctional VE-cadherin and β-catenin and created gaps that were less pronounced than those seen in response to LT (Fig. 3, d and h).

LT, Y-27632, and cytochalasin D caused significant changes in cellular F-actin content and distribution. Control MyEnd cells showed abundant stress fibers throughout the cytoplasm (Fig. 3i), which were largely reduced after treatment with both Y-27632 (Fig. 3j) and cytochalasin D (Fig. 3l). In contrast to
cytochalasin D, which also induced fragmentation of the cortical actin system (as indicated by the arrows in Fig. 3). Y-27632 completely preserved this subset of actin filaments. In fact, the cortical actin staining appeared to be more prominent compared with controls. In LT-treated cells, stress fibers were less numerous but appeared to be thicker and more straight compared with controls (Fig. 3k). At sites of gap formation, the staining of cortical actin filaments was also reduced (see arrows in Fig. 3). This underlines the specificity of LT for Rac-1 as inhibition of Rho would be expected to cause disappearance of stress fibers (as seen in Fig. 3j). These changes in F-actin content were quantified in the present study by phalloidin binding of formaldehyde-fixed monolayers permeabilized with Triton X-100. Compared with control levels, treatment with LT and cytochalasin D resulted in a 50% and 56% reduction of F-actin, respectively. Y-27632 reduced F-actin by only 20% (Table 1).

Effect of LT, toxin B, cytochalasin D, and Y-27632 on VE-cadherin-mediated adhesion probed by laser tweezer. Microbeads coated with VE-cadherin-Fc were allowed to settle for 30 min on MyEnd monolayers before the addition of various compounds. During this period, one of several small filopodial processes was seen to connect individual beads to the endothelial cell surface (Fig. 4a). In control experiments (beads coated with albumin), no attachment structures were seen to develop between the beads and cell surface. In LT-treated cultures, only a few beads could be encountered attached to the endothelium by processes (Fig. 4b). In addition, LT caused the formation of numerous gaps between endothelial cells. As shown recently by immunostaining, typical adherens-type contacts containing VE-cadherin, β-catenin, and F-actin were formed between the beads and surface of endothelial cells (5). In the present study, under control conditions, close to 70% (mean 66%) of beads resisted displacement by laser tweezer,

Fig. 2. LT-induced glucosylation of Rac-1 in mouse myocardial endothelial (MyEnd) cells. A: recombinant RhoA, Rac-1, and Cdc42 were subjected to in vitro glucosylation by LT (200 ng/ml) for 120 min. The degree of glucosylation was determined by [14C]glucose incorporation (10 μM [14C]UDP-glucose) into a 21-kDa band of SDS-PAGE. Glucosylation was performed with and without GDP (100 μM) to enhance glucosylation. Recombinant proteins were subjected to SDS-PAGE. The glucosylation level is expressed in phosphorescence intensity (PI) units. The inset provides two examples of SDS-PAGE (subjected to autoradiography) to show incorporation of [14C]glucose into Rho proteins catalysed by toxin B (100 ng/ml) and LT (200 ng/ml). B: MyEnd cells were treated with LT (200 ng/ml) for 30, 60, 90, and 120 min. The degree of in vivo glucosylation was determined by incorporation of [14C]glucose in cell lysates incubated with 200 ng/ml LT. Cell lysates were subjected to SDS-PAGE and subsequent phosphorimaging. The value of the control level was set to 1.
Fig. 3. Effects of Y-27632, LT, and cytochalasin D on distribution of vascular endothelial cadherin (VE-cadherin) and β-catenin in MyEnd cells. MyEnd cells were treated with Y-27632 (10 μM for 90 min), LT (200 ng/ml for 60 min), and cytochalasin D (10 μM for 50 min). Cells were stained for VE-cadherin (a–d), β-catenin (e–h), and F-actin (i–l), respectively. The distribution of junctional proteins was continuous along the cell borders in control cells (a and e) and unchanged by treatment with Y-27632 (b and f). In contrast, LT (c and g) and cytochalasin D (d and h) caused interruptions of junctional immunostaining and gap formation (arrows), which were far less pronounced in response to cytochalasin D. Scale bar = 20 μm.
whereas the remaining 30% of beads could be removed by laser trapping and thus were considered not specifically bound. In monolayers treated with either LT or cytochalasin D, microbeads resisting displacement by laser tweezer dropped to 55% and 46% of control values (Table 2), respectively. Interestingly, this was about the same value as measured after treatment with toxin B (50%). Inhibition of ROCK by Y-27632 did not significantly increase or decrease the VE-cadherin-mediated bead adhesion (113%) (Table 2). This indicates that the reduction of VE-cadherin binding was mainly caused by inhibition of Rac, because Rac-1 is the only GTPase that is stabilized by jasplakinolide (14). Jasplakinolide caused a significant 25% increase of firmly attached VE-cadherin beads (83% of all beads; Table 3). Subsequent treatment of monolayers with LT in the presence of jasplakinolide caused ~25% of the bound beads to detach (75% of the attached beads remained bound; Table 3) during the after 60 min of treatment. In contrast, jasplakinolide treatment completely abolished bead release by cytochalasin D (101% remained bound; Table 3). As expected, Y-27632 neither caused weakening nor strengthening of bead adhesion in both controls and jasplakinolide-treated cultures. These experiments show that weakening of VE-cadherin-mediated adhesion by LT and cytochalasin D was caused by partial disassembly of the actin filament system or by direct effects of these compounds on the tether between VE-cadherin and F-actin, cells with attached VE-cadherin-coated beads were pretreated with jasplakinolide (10 μM) for 60 min to cause stabilization of F-actin. Jasplakinolide competes with phalloidin for the same binding site on F-actin. Thus actin filaments stabilized by jasplakinolide resist labeling with phalloidin (7). With the use of the phalloidin binding assay, only 7% of control level F-actin was detectable after jasplakinolide treatment completely abolished bead release by jasplakinolide (10 μM). Bound beads were counted and normalized to values of monolayers treated with jasplakinolide only.

Table 1. Depolymerization of F-actin by LT and cytochalasin D is more pronounced than by Y-27632

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-27632 (30 μM)</td>
<td>3</td>
<td>80±3</td>
</tr>
<tr>
<td>LT (200 ng/ml)</td>
<td>3</td>
<td>50±10</td>
</tr>
<tr>
<td>Cytochalasin D (10 μM)</td>
<td>3</td>
<td>56±3</td>
</tr>
<tr>
<td>Jasplakinolide (10 μM)</td>
<td>3</td>
<td>7±1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of cells. Values are normalized to control levels of untreated cells of the same age. The content of F-actin in mouse myocardial endothelial (MyEnd) cells was determined by methanol extraction of bound tetramethylrhodamine isothiocyanate (TRITC)-phalloidin and quantification by fluorescence spectrophotometry. Cells were treated with Y-27632 (10 μM for 90 min), Clostridium sardelli lethal toxin (LT; 200 ng/ml for 60 min), or cytochalasin D (10 μM for 50 min). LT and cytochalasin D drastically reduced the F-actin content, whereas Y-27632 was less effective. The F-actin stabilizing compound jasplakinolide (10 μM for 60 min) blocked virtually all phalloidin binding sites.

Table 2. Reduction of VE-cadherin-mediated adhesion by LT, toxin B, and cytochalasin D but not by Y-27632

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>Bound Beads, %</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21</td>
<td>59–78 (mean = 66)</td>
<td>100</td>
</tr>
<tr>
<td>Y-27632 (30 μM)</td>
<td>9</td>
<td>72±5</td>
<td>113</td>
</tr>
<tr>
<td>LT (200 ng/ml)</td>
<td>6</td>
<td>38±4</td>
<td>55</td>
</tr>
<tr>
<td>Toxin B (100 ng/ml)</td>
<td>6</td>
<td>39±7</td>
<td>50</td>
</tr>
<tr>
<td>Cytochalasin D (10 μM)</td>
<td>6</td>
<td>27±4</td>
<td>46</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of cells. Values are normalized to control levels. Vascular endothelial cadherin (VE-cadherin)-mediated adhesion was assayed by laser trapping of VE-cadherin-coated microbeads attached to the cell surface of MyEnd monolayers. Beads were allowed to settle and bind for 30 min (control values). Afterward, MyEnd monolayers were treated with Y-27632 (10 μM for 90 min), LT (200 ng/ml for 60 min), toxin B (100 ng/ml for 150 min), and cytochalasin D (10 μM for 50 min). In contrast to Y-27632, LT, toxin B, and cytochalasin D caused a significant reduction of bound beads (45–54%).

Table 3. Jasplakinolide blocked the effect of cytochalasin D but not of LT on VE-cadherin-mediated bead binding

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>Bound Beads, %</th>
<th>% of Jasplakinolide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jasplakinolide (10 μM)</td>
<td>11</td>
<td>83±3</td>
<td>100</td>
</tr>
<tr>
<td>Y-27632 (30 μM)</td>
<td>5</td>
<td>80±5</td>
<td>96</td>
</tr>
<tr>
<td>LT (200 ng/ml)</td>
<td>6</td>
<td>62±4</td>
<td>75</td>
</tr>
<tr>
<td>Cytochalasin D (10 μM)</td>
<td>5</td>
<td>84±3</td>
<td>101</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of cells. MyEnd cells were pretreated for 60 min with jasplakinolide and then incubated with Y-27632 (10 μM for 90 min), LT (200 ng/ml for 60 min), and cytochalasin D (10 μM for 50 min) in the presence of jasplakinolide (10 μM). Bound beads were counted and normalized to values of monolayers treated with jasplakinolide only.

Fig. 4. Visualization of structural changes of cell-to-bead contacts in response to LT. Visualization of contacts between endothelial cells and VE-cadherin-coated microbeads by scanning electron microscopy revealed small endothelial processes (inset) attached to the bead surface (a, control). Exposure to LT (200 ng/ml for 60 min) caused a significant reduction of cellular processes attached to the beads (b). Moreover, numerous gaps had formed between endothelial cells. Bar = 10 μm (a), 1 μm (a, inset), and 1 μm (b).
inhibitory action of LT on VE-cadherin-mediated adhesion is independent of F-actin and appears to act directly on the VE-cadherin anchoring complex.

DISCUSSION

The aim of this and our previous study was to analyze the role of Rho family proteins in capillary permeability and barrier function. Toxin B and LT were used as inhibitors of Rho family proteins because our in vitro studies showed that both toxins exert their inhibitory function on endothelial Rho proteins within a time course of 60–150 min. These conditions are applicable for in vivo studies. Whereas toxin B at the concentration used in our previous study (100 ng/ml) (1) inhibited all three Rho family proteins, i.e., RhoA, Rac-1, and Cdc42, we confirmed that the LT preparation used in this study selectively inhibited Rac-1 but not RhoA and only slightly inhibited (<10%) Cdc42. Like toxin B, LT caused a rapid increase of Lp after a delay of 40 min, and this confirms our predictions based on experiments with toxin B and the ROCK inhibitor Y-27632, which leads us to conclude that the toxin B-induced reduction of microvascular barrier properties is mainly due to an inactivation of small GTPases other than RhoA (1). Because LT selectively inhibited Rac-1 but did not significantly inhibit Cdc42 and RhoA, we conclude that Rac-1 plays an essential role in the maintenance of capillary endothelial integrity.

LT action on endothelial adherens junctions. LT has been previously shown to cause disorganization of endothelial junctions as well as final detachment of cells after prolonged treatment for 12–16 h (29). Because LT has been reported to also glucosylate Ras and Ral (8), it is possible that these long-term effects of LT may at least in part be caused by secondary actions on other signaling pathways involved in proliferation and cell survival.

To minimize the possibility of secondary phenomena, we confined our experiments to a short time course of 30–90 min, during which Rac became inactivated by 50–70%. Because the onset of both the increase of capillary permeability and formation of intercellular gaps in MyEnd monolayers became obvious after a lag phase of 30–60 min, we conclude that inactivation of 30–50% of cellular Rac by glucosylation is sufficient for both destabilization of adherens junctions and a significant weakening of adhesion of VE-cadherin-coated beads. Furthermore, LT and toxin B, when used under conditions of comparable Rho protein glucosylation, both reduced binding of VE-cadherin-coated beads by ~50%. The fact that Rac-1 is the only GTPase inhibited by both toxins indicates that mainly inhibition of Rac-1 seems to be causing the described effects. Whether partial inhibition of Cdc42 and of still-undefined other 20- to 30-kDa GTPases might also contribute to barrier breakdown cannot be excluded. Further insight into the role of Rac activity for the maintenance of endothelial barrier properties may come from experiments in which human umbilical vein endothelial cell (HUVEC) monolayers were transfected with recombinant adenoviruses expressing inactive as well as constitutively active Rac mutants (29). Furthermore, inhibition of Rac-regulated intercellular adhesion seems to be involved in barrier breakdown by thrombin in cultured endothelial cells (27).

There are also studies reporting that microinjected active and inactive Rac did not induce gap formation in HUVEC monolayers and that microinjected inactive Rac blocked TNF-α-induced gap formation (6, 26, 28). Although interpretation of these data is difficult because they depend on expression levels and assumed dominant negative effects of the transfected and microinjected proteins, the main conclusion to be drawn is that Rac activity needs to be precisely controlled to maintain endothelial barrier properties. A functional link between VE-cadherin and Rac can be also concluded from studies with VE-cadherin-deficient endothelial cells. Transfection of these cells with VE-cadherin resulted in both the upregulation and membrane recruitment of Rac and the Rac-specific guanosine exchange factor Tiam-1 (20).

Mechanism of LT-induced gap formation and role of the actin cytoskeleton in VE-cadherin-mediated adhesion. The experiments in the present study show that inhibition of Rac by LT has an immediate strong (30–60 min) effect on endothelial junctional integrity as shown by the severalfold increase of capillary permeability and opening of interendothelial junctions in cultured MyEnd cells.

This breakdown of junctional integrity was accompanied at the same time scale by an ~50% reduction of adhesiveness of VE-cadherin-coated beads attached to the free endothelial cell surface. These bead-to-cell contacts can be considered ectopically located adherens-type junctions associated with F-actin, β-catenin, and VE-cadherin (5). Unlike intercellular adhesive junctions, adherens junctions between beads and the free cell surface are not challenged by contractile forces so that contraction-dependent opening of intercellular junctions can be separated from junctional dissociation caused by weakening of cadherin binding.

In MyEnd cells, the main sites of the actin cytoskeleton are intercellular junctions (specialized section of the cortical cytoskeleton) and stress fibers. Treatment with both LT and cytochalasin D caused loss of stress fibers and fragmentations of the cortical actin cytoskeleton. However, exposure to Y-27632 resulted in loss of stress fibers only. The junction-associated actin system became even more prominent in response to Y-27632. It is reasonable to assume that the integrity of the junction-associated actin filament system is important for intercellular adhesion. This would explain why LT and cytochalasin D caused weakening of adhesion, whereas adhesion remained unchanged in response to treatment with Y-27632. Because the adhesive interaction (transinteraction) of VE-cadherin has been shown to be an extremely low-affinity reaction (millimolar range) with a very short half-life of cadherin-cadherin bonds (bonds dissociate almost twice a second), cadherins disconnected from the actin filament cytoskeleton will disappear from junctional sites by rapid lateral diffusion (3, 4). Lateral diffusion of VE-cadherin has been previously shown to increase 10-fold in response to treatment with cytochalasin D (from 0.017 to 0.17 μm²/s), and this increase of mobility was accompanied by a dispersal of cadherin clusters (5).

LT-induced weakening of bead adhesion was only partly caused by an LT-induced loss of F-actin, as demonstrated by experiments in which cellular F-actin was stabilized by pretreatment with jasplakinolide. Whereas cytochalasin D-induced weakening of VE-cadherin-mediated adhesion was completely blocked by jasplakinolide pretreatment, ~50% of anti-
adhesive activity of LT was independent of F-actin. This indicates that LT-induced inhibition of Rac exerts an additional direct effect on cadherin-mediated adhesion that is independent of the integrity of the actin filament cytoskeleton. Disconnection of cadherins from the actin filament system and depolymerization of F-actin are probably the two main mechanisms of how LT-induced inhibition of Rac leads to untethering of VE-cadherin, followed by disassembly of endothelial adherens junctions and subsequent endothelial barrier breakdown.

ACKNOWLEDGMENTS

We are grateful to Gabriele Lang, Agnes Weth, and Joyce Lenz for skillful technical assistance.

GRANTS

This study was supported in part by National Heart, Lung, and Blood Institute Grants HL-44485 and HL-28607 and by Deutsche Forschungsgemeinschaft Grant SFB 487 (TP B5).

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