Endothelial Rho signaling is required for monocyte transendothelial migration

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Abstract Bacterial toxins affecting Rho activity in microvascular endothelial cells were employed to elucidate whether endothelial Rho participates in regulating the migration of monocytes across monolayers of cultured endothelial cells. Inactivation of Rho by the *Clostridium* C3 exoenzyme resulted in an increased adhesion of peripheral blood monocytes to the endothelium and a decreased rate of transendothelial monocyte migration. Cytotoxic necrotizing factor 1-mediated activation of endothelial Rho also reduced the rate of monocyte transmigration, but did not affect monocyte–endothelium adhesion. Thus, efficient leukocyte extravasation requires Rho signaling not only within the migrating leukocytes but also within the endothelial lining of the vessel wall.

Key words: Actin cytoskeleton; Bacterial toxin; Cell–cell contact; Leukocyte extravasation

1. Introduction

The directed migration of leukocytes across the vascular endothelium into inflamed tissue is mediated through a cascade of leukocyte–endothelium interactions. These range from an initial contact resulting in leukocyte capturing to firm adhesion of the leukocytes on the apical side of the endothelium and finally to the transendothelial migration of the leukocytes which is thought to require at least in some cases a regulated opening and closure of the interendothelial cell–cell contacts (for review see [1]). It is well established that the cytoskeleton of the leukocytes is subject to marked rearrangements during this migration process. Particularly pronounced are changes in the actin cytoskeleton that occur during leukocyte adhesion, polarization and migration in chemotactic gradients. Key regulators of these actin rearrangements are Rho GTPase family members (for review see [2–4]). Among other things it has been shown that Cdc42 and Rac1 participate in the regulation of leukocyte cell polarity, lamellipodia formation and directed migration and that monocyte transmigration through an endothelial monolayer requires Cdc42 activity [5–7]. Moreover, RhoA is specifically required for monocyte tail retraction during transendothelial migration and a RhoA/Rho kinase (ROCK)-dependent signaling pathway is involved in triggering the rear end detachment of migrating neutrophils [8,9]. Mouse knockout models also support the role of Rho family members in leukocyte migration since, e.g. targeted disruption of the hematopoietic cell-specific Rac2 causes general defects in F-actin rearrangements and chemotaxis in mouse neutrophils [10].

In contrast to the clearly documented role of Rho family members in leukocyte locomotion less is known about the Rho-dependent regulation of the endothelial cytoskeleton during leukocyte passage. While the endothelial actin-cytoskeleton and actomyosin dynamics regulated by myosin light chain kinase have been shown to participate in regulating neutrophil and monocyte transendothelial migration [11–15] Rho family members have been linked mainly to the regulation of endothelial cell–cell contacts. By using bacterial toxins inactivating Rho proteins as well as by employing dominant-negative Rho mutants and Rho kinase inhibitors Rho and Rac were shown to regulate endothelial cell permeability and barrier function [16–19]. At least in part this regulation could reflect a role of Rho family members in regulating cadherin-mediated cell–cell adhesion which has been documented more precisely in epithelial cells (for review see [20]). A role of Rho GTPases in regulating transendothelial lymphocyte traffic has been deduced from experiments analyzing the migration of lymphocytes through brain endothelial cell layers of high electrical resistance which revealed an inhibitory effect of Rho inactivating toxins [21].

To establish whether endothelial Rho family members also participate in regulating leukocyte migration through microvascular endothelium, i.e. the site of the vasculature where most leukocyte extravasation is occurring, we made use of a cell culture model reproducing this transmigration event and employed bacterial toxins to interfere with Rho activity. We show that inactivation of endothelial Rho by *Clostridium limosum* exoenzyme C3-triggered ADP ribosylation results in a reduced rate of monocyte transmigration through monolayers of microvascular endothelial cells. A similar effect is observed when endothelial stress fibers are stabilized by activating Rho through deamidation of Gln-63 by *Escherichia coli* cytotoxic necrotizing factor CNF1. Altering the state of active Rho has no (CNF1) or a stimulatory effect (C3) on monocyte adhesion indicating that intraendothelial events required for both monocyte adhesion and subsequent transendothelial migration are regulated through Rho-dependent signaling.

2. Materials and methods

2.1. Cells

Monocytes were isolated from human buffy coats by Ficoll-Paque

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and subsequent Percoll density gradient centrifugation [22]. Cells were then kept overnight in McCoy's 5a medium containing 15% fetal calf serum (FCS) at 37°C in 5% CO_2 using hydrophobic Teflon bags and used for transmigration or adhesion assays the next day.

Human dermal microvascular endothelial cells (HMEC-1), kindly provided by Dr. F. Candal (Centers for Disease Control, Atlanta, GA, USA), were cultured at 37°C in 3% CO_2 in MCDB131 supplemented with 10% FCSgold (PAA Laboratories, Cölbe, Germany)) and 20 mM L-glutamine, 50 μg/ml (at least 30 units/ml) gentamicin, 10 ng/ml epidermal growth factor, and 1 μg/ml hydrocortisone.

2.2. Bacterial toxins

The *E. coli* cytotoxic necrotizing factor (CNF1) was purified as described [23], lyophilized and dissolved in sterile H_2O to prepare a 1 mg/ml stock solution. Prior to the actual experiment this stock solution was diluted in HMEC-1 culture medium to yield a final concentration of 500 ng/ml. The *C. limosum* C3 exoenzyme could not be used directly as it is not taken up by intact endothelial monolayers. Therefore we made use of a fusion toxin (C2IN-C3) comprising the entire C3 exoenzyme fused to the N-terminal part of the C2I component of the *Clostridium botulinum* C2 toxin. In conjunction with the membrane binding C2II protein the latter mediates cellular uptake of the C2IN-C3 fusion toxin [24]. The C2IIa and the C2IN-C3 fusion toxin (stock solutions at 100 μg/ml each) were added to endothelial monolayers at a concentration of 200 ng/ml, and the monolayers were then incubated with the toxins for different times (between 2 and 16 h). Subsequently, cells were washed and then kept in toxin-free medium for the different assays. In one series of experiments the C2IN-C3 fusion toxin was present throughout the entire transmigration assay to elucidate the effect of the toxin on the monocytic Rho in our experimental setup.

2.3. Transmigration assay

Transmigration assays were performed essentially as described previously [25]. Briefly, 2.2×10^5 HMEC-1 were seeded on fibronectin-coated 6.5 mm Transwell filters with a 5 μm pore size and grown to confluency. After 48 h, medium and non-adherent cells were removed, and 600 μl assay medium (MCDB131 supplemented with 10% FCSgold and 20 mM L-glutamine, 50 μg/ml (at least 30 units/ml) gentamicin) were added to the lower compartment of a two-chamber system separated by the Transwell filters. 2×10^6 monocytes in 100 μl assay medium were added to the upper chamber, and cells were subsequently incubated at 37°C and 3% CO_2 for 2 or 4 h. To analyze the effect of Rho activation or inactivation, endothelial cells were preincubated for the times indicated in medium containing the bacterial toxins. Before the addition of monocytes the toxin-containing medium was replaced by fresh assay medium. Cells which had transmigrated through the endothelial monolayer were recovered in the lower tissue culture chamber and quantified by counting in a Coulter Counter Z2 (Coulter, Krefeld, Germany). Data are presented as percentages of cells transmigrated across a toxin-treated as compared to a non-treated monolayer. To verify the integrity of the endothelial monolayer after the assay, the upper chamber was washed twice with PBS, fixed, stained with Diff Quick (Dade Behring, Düdingen, Switzerland), air dried and mounted on glass slides for microscopical analysis. Experiments were routinely carried out in quadruplicate.

2.4. Analysis of monocyte–endothelium adhesion, endothelial permeability and transendothelial resistance

Monocyte adhesion to the toxin-treated vs. non-treated endothelium was analyzed by quantifying myeloperoxidase activity of adherent monocyte as described previously [14]. Moreover the toxin effect on endothelial barrier properties was elucidated by measuring both, the transendothelial permeability towards macromolecules (HRP) and the transendothelial electrical resistance as described [25].

2.5. Statistics

All data were evaluated by a Student's *t*-test and *P* values ≤ 0.01 were considered significant. All error bars represent standard errors.

2.6. Immunofluorescence

The effect of the Rho-manipulating toxins on the actin cytoskeleton and endothelial junction proteins was analyzed in HMEC-1 grown to confluency on fibronectin-coated coverslips. Following toxin treatment cells were rinsed in PBS and then fixed in 3.7% formaldehyde in PBS for 20 min at room temperature. Subsequently, cells were permeabilized for 5 min at room temperature in 0.2% Triton X-100 in PBS, washed in PBS and subjected to labeling with rhodamine-conjugated phalloidin for 30 min at 37°C or stained with primary antibodies directed against ZO-1, cadherin-5 (VE-cadherin) or β-catenin (purchased from BD Transduction Laboratories). Following washing primary antibodies were labeled by treatment for 30 min at 37°C with the respective secondary antibodies coupled to FITC. Cells were washed again and then mounted in mowiol with 4% *n*-propylgallate as antifade agent. Microscopic inspection employed a DM RXA fluorescence microscope (Leica, Wetzlar, Germany) connected to a cooled CCD camera.

3. Results and discussion

3.1. Effects of Rho activation and inactivation on the endothelial actin cytoskeleton and components of interendothelial junction complexes

Bacterial toxins have provided a powerful means of altering in a specific manner the activity of Rho GTPases (for review see [26,27]). The C3 exoenzyme of *C. limosum* has been of particular interest since it inactivates Rho by ADP ribosylation at asparagine 41 without affecting other members of the Rho superfamily like Rac and Cdc42 [28]. Constant activation of Rho can also affect its dynamic cycling between the GTP and GDP bound conformation and can thereby interfere with a regulatory function of Rho. Such activation can be achieved by *E. coli* cytotoxic necrotizing factor (CNF) 1 which deamidates glutamine 63 of Rho [23]. Thus we decided to specifically manipulate Rho in microvascular endothelial cells by employing the C3 protein toxin and CNF1 in order to elucidate the importance of Rho signaling on leukocyte–endothelium interactions and leukocyte transendothelial migration.

First, we needed to establish that the bacterial toxins had the anticipated effects on the endothelial actin cytoskeleton. Monolayers of the microvascular endothelial cell line HMEC-1 were subjected to toxin treatment for different times and subsequently stained with rhodamine–phalloidin to visualize F-actin. Since the C3 exoenzyme does not penetrate the plasma membrane the experiments employed a C2IN-C3 fusion toxin in which C3 had been fused to the N-terminal part of the *C. botulinum* C2I toxin thereby enabling cellular uptake when used in conjunction with the membrane binding C2II protein [24]. Fig. 1 (upper panels) reveals that Rho inactivation or activation in HMEC-1 by C2IN-C3 or CNF1, respectively, resulted in a significant loss (C2IN-C3) or a marked increase (CNF1) in actin stress fibers. The effects of both toxins were reversible and the appearance of the actin cytoskeleton returned to normal following removal of the toxins and incubation of the HMEC-1 in toxin-free medium for a period of at least 4 h (not shown).

Given these effects on the actin cytoskeleton we next analyzed whether Rho inactivation or activation affected the localization of proteins of endothelial junctions which are linked directly or via adapter proteins to the actin cytoskeleton. Neither ZO-1, a component of tight junctions, nor VE-cadherin or β-catenin, which are found in adherens junctions, are altered following C2IN-C3 treatment and stress fiber reduction. Rho activation by CNF1 also has no marked effect on the localization of the junctional markers although the ZO-1, VE-cadherin and β-catenin stainings seem to be more pronounced in the CNF1-treated cells (Fig. 1, lower panels). Thus, although Rho family GTPases have emerged recently as regulators of cadherin-mediated cell–cell adhesion, it appears

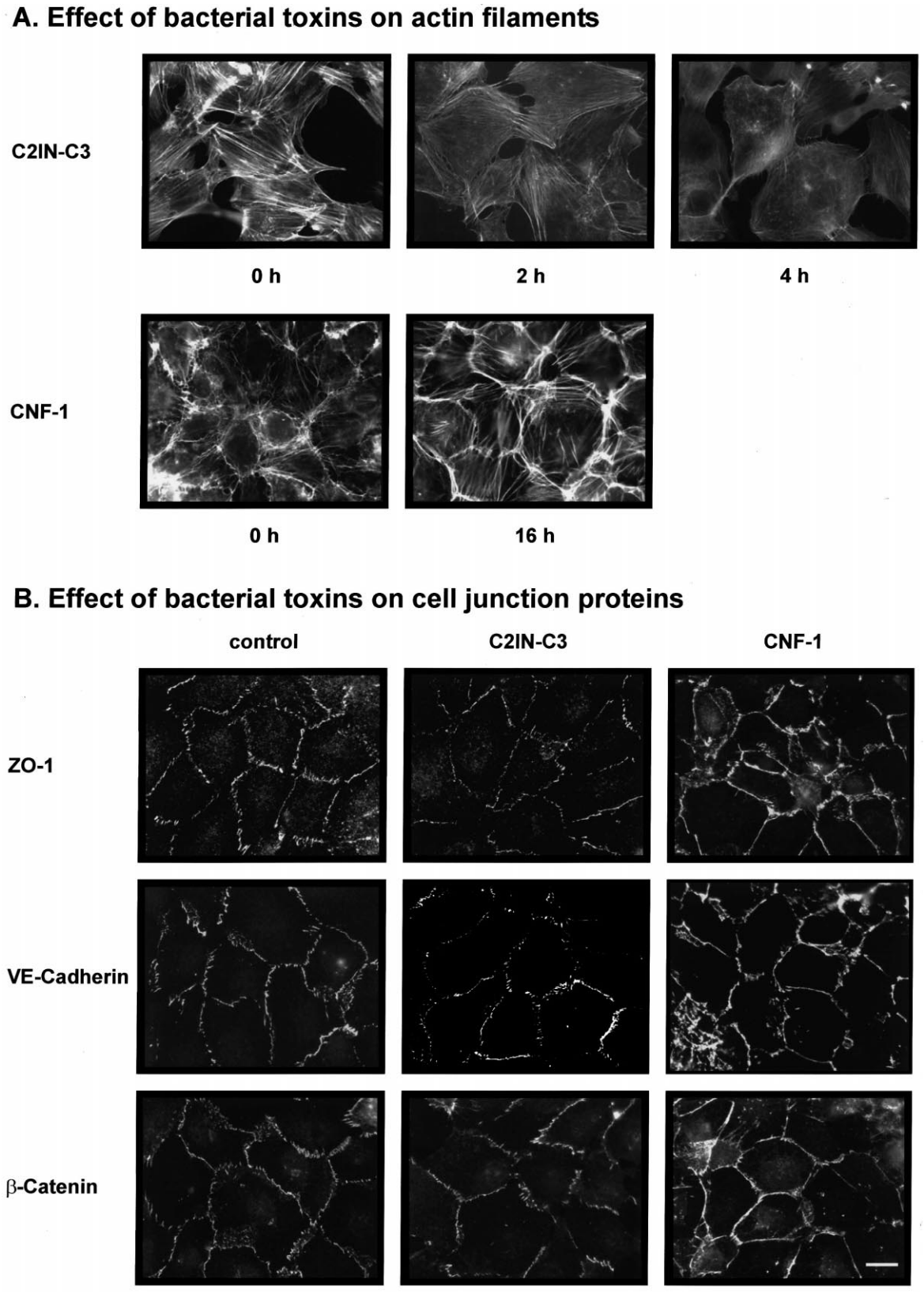

Fig. 1. Effect of Rho inactivation or activation on endothelial F-actin and components of interendothelial junctions. A: HMEC-1 monolayers were incubated with the C2IN-C3 or CNF1 toxins, as described in Section 2. At the times indicated cells were fixed and processed for rhodamine–phalloidin staining to visualize F-actin. B: HMEC-1 were treated with the C2IN-C3 or CNF1 toxins for 3 and 16 h, respectively. Subsequently, cells were fixed and processed for indirect immunofluorescence using antibodies against the cell–cell junction proteins ZO-1, VE-cadherin, and β-catenin. Scale bar represents 10 μm.

that in HMEC-1 Rho itself has no or only a minor effect on the appearance of interendothelial junctions. However, although the junction components analyzed appear unaffected or hardly affected in their localization over the entire time course of toxin treatment we cannot exclude a more subtle effect on the dynamics of adherens junctions which is not evident in our morphological analyses. Moreover, it remains possible that Rac1 and Cdc42 are more important for regulating endothelial adherens junctions than RhoA which is targeted by the toxins in our experimental setup (for review see [20]).

3.2. The transendothelial migration of monocytes is affected by Rho inactivation or activation

To elucidate whether endothelial Rho participates in regulating endothelium–leukocyte interactions and/or the transendothelial migration of leukocytes, endothelial monolayers were pretreated with the bacterial toxins for 3 h (C2IN-C3) or 16 h (CNF1), and then subjected to monocyte adhesion and transmigration assays. Moreover, the barrier function of the toxin-treated endothelial cells was characterized by measuring macromolecule flux across endothelial monolayers. Duration of the toxin pretreatment and concentration of the toxin employed were chosen to have the maximum stabilizing or destabilizing effect on the endothelial F-actin (see Fig. 1). Moreover, we verified through wash-out experiments that the toxin effects, i.e. destabilization or stabilization of stress fibers, persisted in HMEC-1 pretreated with the toxins and kept in toxin-free medium for the duration of our adhesion (30 min) and transendothelial migration assays (2–4 h), respectively (not shown).

Fig. 2 summarizes the results obtained following Rho inactivation through the C2IN-C3 fusion toxin which was introduced via the C2IIa membrane binding component. In the experiments shown toxin-treated endothelial cells are compared to non-treated control cells since a series of control experiments had established that administration of the membrane binding C2IIa component alone had no significant effect on endothelial permeability, leukocyte adhesion and transmigration (not shown). Moreover, previous experiments had revealed that C2IN itself is enzymatically inactive and that the individual toxin components (C2IN-C3 and C2IIa) have no effect on cell morphology [24,29]. Pretreatment of the endothelial cells with both, the C2IN-C3 fusion toxin and the C2IIa membrane binding component, which leads to a destabilization of endothelial F-actin (see Fig. 1), induces a significant increase in the adhesion of monocytes (Fig. 2, 3 h column). An even greater stimulation of monocyte adhesion is observed when the C2IN-C3 and C2IIa toxins were also present during monocyte adhesion thereby affecting both, endothelial and monocytic Rho (Fig. 2, 3 h+1 h column). The stimulatory effect is less pronounced when the endothelial cells were activated by cultivation in the presence of TNF-α for 16 h prior to the toxin treatment. Under these conditions the TNF-α-induced increase in adhesion was approximately 110% in the case of untreated control cells, 55% for HMEC-1 pretreated with the toxin for 3 h and 35% in cases where adhesion was analyzed in the presence of toxin (3 h+1 h; not shown). Taken together, this indicates that adhesion molecules mediating leukocyte–endothelium interactions under the conditions chosen, i.e. integrins on monocytes and ICAM-1 and VCAM-1 on the endothelium [14], require

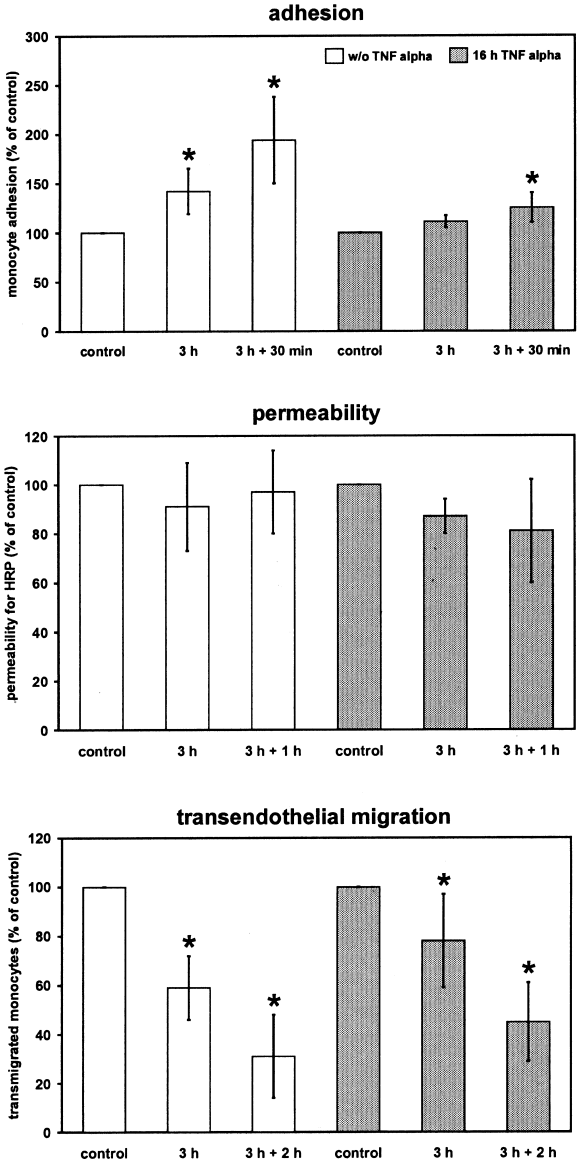

Fig. 2. Inactivation of endothelial Rho by C2IN-C3 affects monocyte–endothelium adhesion and monocyte transendothelial migration. HMEC-1 were pretreated with the C2IN-C3 toxin for 3 h and then subjected to permeability, monocyte adhesion and transmigration assays in either toxin-free medium (3 h columns) or in the presence of toxin (3 h+30 min, 3 h+1 h and 3 h+2 h columns, respectively). The latter experimental conditions were chosen to inactivate Rho proteins in both, endothelial cells and monocytes. In each assay results obtained for the toxin-treated cells were compared to those of control cells kept for the same time in toxin-free medium.

Rho activity for optimal presentation on the cell surface and that inactivation of Rho results in a higher and possibly aberrant surface concentration of interaction-competent adhesion molecules.

A contrasting and even more significant effect is observed when the migration of monocytes through C2IN-C3-treated HMEC-1 monolayers is analyzed (Fig. 2). While the transendothelial permeability remains unaffected, toxin treatment results in a reduction of the rate of transmigration as compared to that through control monolayers. The effect is observed for both, unstimulated and TNF-α-stimulated HMEC-1 although the reduction is somewhat less pronounced in the latter case.

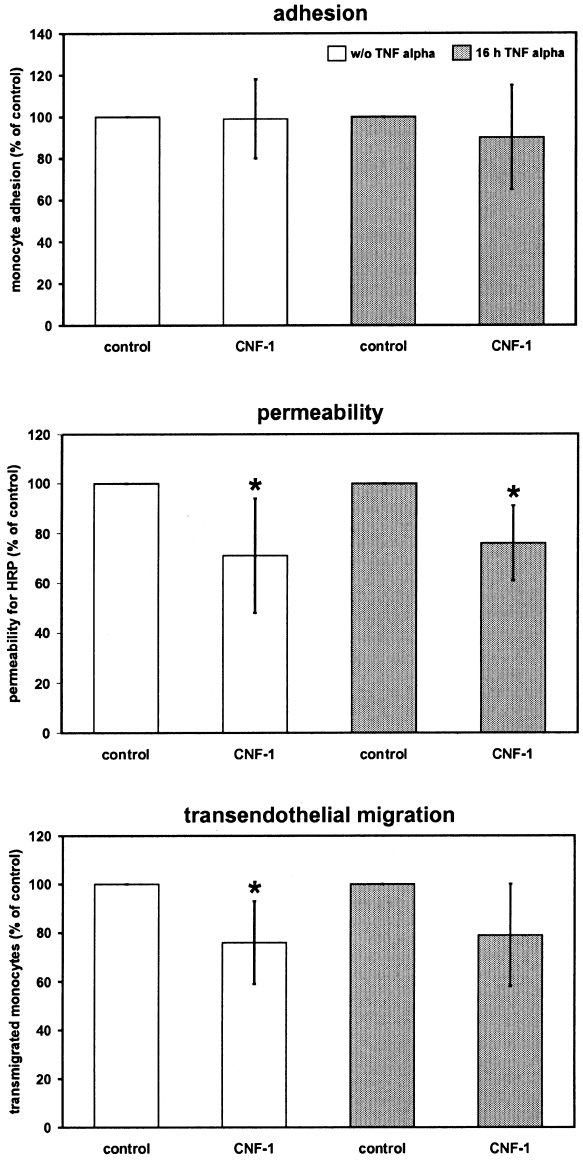

Fig. 3. CNF1-mediated activation of endothelial Rho increases endothelial barrier properties and decreases monocyte transmigration. HMEC-1 monolayers were treated with CNF1 toxin for 16 h and then subjected to permeability, monocyte adhesion and transmigration assays in toxin-free medium. Control cells were kept in toxin-free medium for the duration of the pretreatment and values obtained for these controls were set to 100%.

This could suggest that the TNF-α-induced expression of endothelial adhesion molecules, which is required for efficient transmigration, could bypass to some extent a need for Rho signaling. The inhibitory effect of the *Clostridium* toxin on transendothelial migration is even more pronounced when the toxin is also present during the actual course of the transmigration assay thereby also affecting Rho in the migrating monocytes. Since Rho signaling is known to be required for cell migration (for review see [2–4]) the strong inhibition seen in these experiments is expected and thus serves as a positive control for the validity of our assay.

In contrast to C3-mediated Rho inactivation, activation of endothelial Rho by CNF1 treatment has no effect on monocyte adhesion. The transmigration of monocytes through CNF1-treated endothelial monolayers, however, is slightly reduced as compared to non-treated controls and this reduction is observed for both non-stimulated and TNF-α-stimulated endothelial cells (Fig. 3). Moreover, CNF1 treatment results in a decreased transendothelial permeability of macromolecules (Fig. 3) and an increased transendothelial resistance (not shown). Thus in line with the somewhat stronger concentration of endothelial junction proteins in CNF1-treated endothelial monolayers (see above) the toxin-mediated activation of Rho appears to trigger a stronger interendothelial adhesion with a resultant reduction in transendothelial permeability and cell migration.

At present, it is not known whether in our cell system Rho acts directly on components of interendothelial junctions thereby affecting the tightness of cell–cell contacts or whether it acts through regulation of the actin cytoskeleton dynamics and/or the actin cytoskeleton/junction interface. Proteins interacting with and regulating Rho have been described in epithelial cell–cell junctions, e.g. p120/catenin which inactivates the intrinsic guanine nucleotide exchange activity of RhoA thereby causing inactivation (for review see [20]), but it is not known whether such interactions are of functional relevance for the Rho-dependent regulation of endothelial barrier functions. Subtle or perhaps even more pronounced differences between the cell–cell contacts of different types of cells can probably be expected and can explain the differing effects of C3-mediated Rho inactivation on the recruitment of cadherin to intercellular junctions. While injection of C3 into keratinocytes inhibits the accumulation of cadherin at cell–cell adhesion sites induced in the presence of calcium [30], introduction of the toxin into endothelial monolayers does not affect the junctional localization of VE-cadherin or the endothelial barrier formation (see Figs. 1 and 2). Future experiments have to reveal whether such differences are due to a specific junction arrangement in microvascular endothelial cells and/or reflect an altered Rho requirement in developing as compared to established cell–cell contacts.

3.3. Conclusions

Bacterial toxins were employed to alter Rho activity in monolayers of microvascular endothelial cells. While Rho activation results in an increased endothelial barrier formation, Rho inactivation has no effect on the transendothelial permeability or the localization of proteins found in tight and adherens junctions. However, interfering with Rho dynamics by permanently activating or inactivating the GTPase reduces the rate of migration of blood monocytes through the toxin-treated endothelial monolayers. Since actin cytoskeleton dynamics in the endothelium are thought to be required for efficient leukocyte transmigration, the experiments point towards an important role of endothelial Rho in regulating these dynamics. Thus, not only the Rho GTPase-regulated cytoskeleton of migrating leukocytes but also that of the endothelial layer of the microvasculature appears to play an active role in supporting leukocyte extravasation.

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