7 General Discussion

una mirada no dice nada
y al mismo tiempo lo dice todo
como la lluvia sobre tu cara
o el viejo mapa de algun tesoro

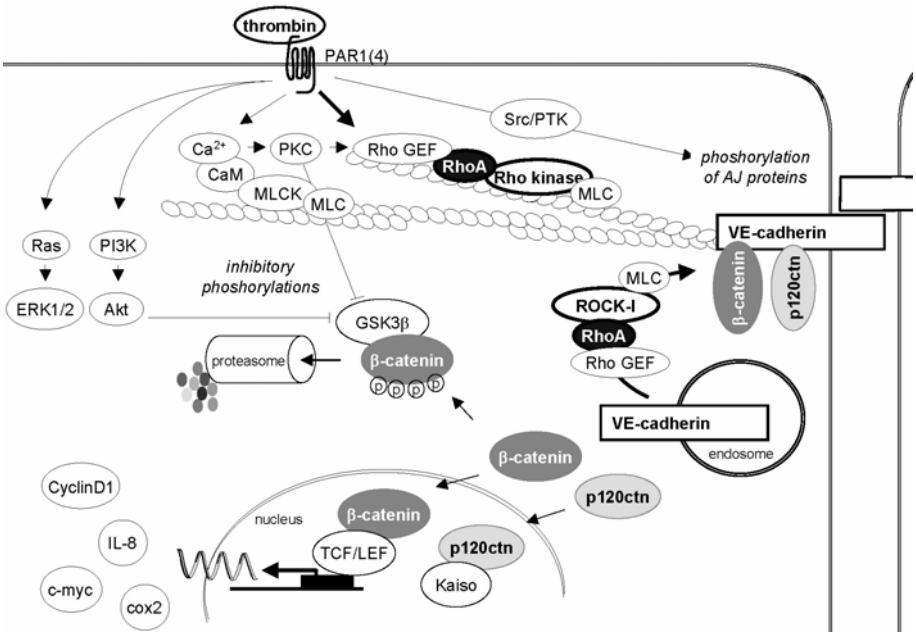
Endothelial cells (ECs) represent the inner lining of blood vessels. They form a physical barrier that actively regulates the exchange of nutrients and macromolecules between the blood and the surrounding tissue. In this thesis we addressed the role of RhoA and Rho kinase in different aspects of endothelial barrier regulation. In addition we investigated the role of β-catenin and p120ctn, two proteins associated with adherens junctions (AJs), in endothelial permeability-related gene expression. The aim of our studies was to unravel how RhoA/Rho kinase and the mutual interactions between the AJs and RhoA activity contribute to endothelial barrier function, and to determine whether thrombin-induced AJ disassembly affects β-catenin-mediated gene expression.

In short, we showed a dual and opposing role for RhoA/Rho kinase in the regulation of the endothelial barrier function. The intrinsic activity of RhoA/Rho kinase is involved in maintaining endothelial barrier integrity, whereas thrombin-stimulated RhoA/Rho kinase activity is involved in endothelial barrier disruption (Fig. 1). Our *in vitro* and *in vivo* data suggested that thrombin-induced endothelial permeability is primarily ROCK-II dependent. However, for a full-blown thrombin-response ROCK-I is also required. In addition, we demonstrated a time and location dependent role for RhoA in thrombin-mediated endothelial permeability. In contrast to the widely accepted view that RhoA activation occurs all around the cell at the plasma membrane, our studies revealed that thrombin-mediated RhoA activation in EC monolayers occurs at small fin-like membrane ruffles, as well as at SFs, where it is active during fiber remodeling (see Chapter 5, Fig. 6). Furthermore, we reported that this thrombin-induced Rho/Rho kinase-mediated interendothelial gap formation is accompanied by a membrane to cytosol relocalization of β-catenin as well as p120ctn. Simultaneously, thrombin inhibits the cytosolic degradation of β-catenin, thereby allowing nuclear translocation of active β-catenin resulting in increased expression of β-catenin inducible genes (Fig. 1).

In this general discussion we will place our findings in the context of the rapidly developing field of vascular permeability, discuss limitations of our studies and indicate aspects that should receive future attention. We will first address the role of Rho kinase, and subsequently its two isoforms, in endothelial barrier regulation. Second we will discuss implications of the subcellular localizations of RhoA activity in EC monolayers under non-stimulated and thrombin-stimulated conditions and finally we will reflect on our findings regarding permeability-induced nuclear relocalization of β-

catenin, its effect on gene expression and the implications for vascular biology.

Figure 1. Model for thrombin-induced permeability in ECs.



Thrombin stimulation of ECs activates several pathways, including the Rho/Rho kinase-mediated acto-myosin interaction and SF formation. Rho/ROCK-II facilitates the contraction force that, in combination with protein tyrosine kinase (PTK)-induced phosphorylation, causes the disassembly of the AJ. This induces interendothelial gap formation and the relocalization of β-catenin and p120ctn from the membrane to the cytosol. Cytosolic β-catenin via GSK3β-induced phosphorylation and ubiquitination, is normally degraded by proteasomes. Thrombin mediated inhibition of GSK3β however, stabilizes cytosolic β-catenin allowing nuclear translocation and subsequent β-catenin-mediated pro-angiogenic gene expression. By binding Kaiso, a gene repressor, p120ctn may play an important role in this process. The recovery of the endothelial barrier may involve Rho/ROCK-I-mediated recycling of VE-cadherin.

Dual role of Rho kinase in endothelial barrier regulation

Since the late 1990s the importance and roles of Rho GTPases in regulating the endothelial barrier function are being discovered. This thesis and work of others have revealed that the spatio-temporal regulation of RhoA, Rac1 and Cdc42 is crucial for the regulation of the endothelial barrier. Activation of RhoA and its best-known downstream target Rho kinase by vasoactive

agents is known to increase SF-mediated cell contraction, focal adhesion (FA) formation and AJ disassembly resulting in interendothelial gap formation.[1-6] In **chapter 3** we investigated the contribution of basal Rho kinase activity to endothelial barrier regulation. We showed for the first time an intrinsic Rho kinase activity (as measured by phosphorylation of MYPT1 and MLC-2) at the cell periphery of confluent ECs, which co-localizes with the cortical F-actin cytoskeleton. Under these conditions Rho kinase activity in the cytosol had a punctated pattern that did not co-localize with F-actin. To our surprise, thrombin subsequently decreased the Rho kinase activity at the cell periphery whereas it induced an increase at cytosolic SFs. These data indicate a dual role for Rho kinase activity during control and thrombin-stimulated conditions. We have confirmed these data in intact microvessels.

We next showed that the basal Rho kinase activity is involved in maintaining endothelial barrier integrity. Inhibition of Rho kinase with several chemically unrelated pharmacological inhibitors (Y-27632, fasudil, H1152) and short interfering (si) RNA against both isoforms of Rho kinase, ROCK-I and ROCK-II, without affecting RhoA and Rac1 activities, eNOS expression or apoptosis, increased the endothelial permeability as measured by passage of horseradish peroxidase (HRP) and transendothelial resistance (TEER) over ECs cultured on porous filters.

In addition, Rho kinase inhibition decreased the peripheral phosphorylation levels of MYPT1, MLC-2 and the amount of VE-cadherin, which respectively indicate that the activity of Rho kinase, the subsequent tension development needed for proper barrier function and AJ formation, as well as VE-cadherin expression decreased. Combined with literature data showing that the interactions of junctional complexes with F-actin are very dynamic[7-10] and that the endothelial barrier is enforced by Rho kinase-mediated contractile forces that mediate the recycling of VE-cadherin-containing vesicles to the plasma membrane,[11, 12] these data suggest that intrinsic Rho kinase activity maintains endothelial barrier integrity by mediating the recycling of VE-cadherin to the junctional complexes. At the same time, Katoh *et al*.[13] reported in cultured fibroblasts that short Rho kinase inhibition with Y-27632 hardly affects peripheral actin filaments and FAs, whereas in the cell body they are clearly decreased. Furthermore, the Rho-kinase-dependent organization of FAs occurred independently of the formation of SFs. In line with our data, they suggested that Rho-kinase directly or indirectly influences the accumulation of the FA-associated proteins during the organization of the FAs. Taken together, this suggests that the dual role of Rho kinase in barrier integrity is not restricted to ECs.

In **chapter 3** we also showed that whereas the knockdown of ROCK-II did not affect barrier integrity, the knockdown of ROCK-I might slightly enforce the barrier. Soon after publication of these data, Samarin *et al.*[14] reported that pharmacological inhibition of Rho kinase with Y-27632 and H1152 prevented Ca^{2+}-depletion-induced AJ disassembly in epithelial cells. Moreover, they proved that this process was ROCK-II- but not ROCK-I-dependent and that the recruitment of the Rho activating protein GEF-H1 to cortical F-actin is involved in this process. Subsequently, Iwanicki *et al*.[15] provided evidence in fibroblasts, in which ROCK-I activity is higher then ROCK-II activity,[16] that tail retraction upon lysophosphatidic acid (LPA) stimulation is ROCK-II-dependent and that this pathway involves PDZ-RhoGEF-mediated RhoA activation. In contrast, in lung ECs Mong and Wang[17] recently showed that ROCK-II activity is greater then ROCK-I activity and that although stimulation of the ECs activates both ROCK-I and ROCK-II, ROCK-I is required for early permeability increases by TNFα. Another study showed that in ECs thrombin activates mainly ROCK-II.[18] Taken together, evidence for a differential role for ROCK-I and ROCK-II in endothelial barrier regulation was accumulating. In **chapter 4**, we therefore investigated the role of ROCK-I and ROCK-II in thrombin-induced endothelial permeability.

In addition to the isoform specific siRNAs against ROCK-I and ROCK-II we used new pharmacological inhibitors that were provided by Surface Logix (Brighton, USA) and compared their effect to the effect of Y-27632. The drug Slx-2119 is ROCK-II specific,[19] whereas Slx-3242 inhibits both ROCK-I and ROCK-II. Regarding ROCK-I, our data illustrate that although only inhibition of both isoforms increased basal permeability, the knockdown of ROCK-I slightly enhanced thrombin-mediated permeability. We thus propose that ROCK-I is involved in endothelial barrier maintenance by recycling of VE-cadherin. Additionally, we showed that thrombin-mediated ERM phosphorylation, as well as thrombin-induced permeability as measured by HRP passage, TEER and electrical cell substrate impedance sensing (ECIS), is mainly ROCK-II dependent. Morphological analysis of thrombin-stimulated ECs further revealed that ROCK-II is not required for thrombin-mediated SF formation, which is only prevented by the inhibition of both Rho kinase isoforms. Interestingly, Ivanov *et al.*[20] recently reported that in epithelial cells stimulation of protein kinase C via ROCK-II-dependent activation of MLC-2, which increases the contractility at the cortical F-actin structures, disrupts the junctional complexes. Our *in vitro* data were verified in siRNA-treated mice by measuring Evans blue extravasation and wet-dry

ratios of isolated lungs after *in vivo* stimulation with the thrombin receptor activating peptide (TRAP).
In conclusion, endothelial barrier maintenance may depend on Rho kinase (possibly ROCK-I)-mediated recycling of VE-cadherin to the plasma membrane (Fig.1). Stimulus-dependent disruption of the endothelial barrier involves Rho GEF-mediated activation of RhoA and subsequent Rho kinase activation to enhance MYPT-1 and MLC-2 phosphorylation resulting in enhanced contractility, AJ disruption and interendothelial gap formation (Fig. 1).
In terms of therapeutic value, if Rho kinase is involved in both barrier disrupting and barrier protective processes, then prolonged inhibition of Rho kinase may have negative therapeutic effects. Rho kinase targeting agents may be helpful during acute vascular leakage, but might aggravate chronic conditions.
The next step may thus be to investigate whether ROCK-I *in vivo* enforces the endothelial barrier and whether it is possible to increase the activation of ROCK-I that may enforce endothelial barrier integrity. Furthermore, we would want to know whether *in vivo* vascular permeability is indeed dependent on ROCK-II and if so, whether targeting of only ROCK-II prevents negative therapeutic effects on the long term.

Dual role of RhoA in endothelial barrier regulation

When we started the work for this thesis, studies had elucidated an important contribution of RhoA to prolonged endothelial permeability.[2, 3, 21-24] However, the exact mechanisms by which RhoA activation contributes to the endothelial permeability response and its applicability *in vivo* were largely unknown. To clarify which cell structures are associated with RhoA activity during endothelial barrier disruption, we determined the subcellular localization of RhoA activation under non-stimulated and thrombin-stimulated conditions. Fluorescent resonance energy transfer (FRET) microscopy, which enables the visualization of RhoA activation in real-time (live), was performed using Raichu-RhoA probes that were a kind gift from Prof.dr. M. Matsuda (Osaka University, Japan).
We first demonstrated that in confluent EC monolayers under non-stimulated conditions RhoA was activated prior to gap closure and was additionally active at membrane ruffles. This clearly indicates that like Rho kinase, RhoA plays a role in endothelial barrier maintenance. In terms of downstream signaling it is of interest that Rho-mediated membrane ruffling under non-

stimulated conditions depends on mDia signaling,[5, 25-28] whereas, as shown above, the Rho-mediated gap closure most likely involves Rho kinase- (possibly ROCK-I) mediated VE-cadherin recycling to the plasma membrane.

We subsequently investigated the role of RhoA in thrombin-mediated endothelial barrier disruption. RhoA activity was determined during the first 15 minutes of thrombin stimulation. Thrombin-mediated a time- and location-dependent activation of RhoA followed by cell contraction and interendothelial gap formation that was prevented by the RhoA inhibitor C3 transferase. We showed that thrombin induced a rapid (<30 sec) and transient (~4 min) increase in RhoA activity at the membrane, particularly at small fin-like ruffles.

The finding that RhoA activation occurs at the membrane is in line with pull-down assays for active RhoA performed by others[29-32] and us,[33, 34] which evidence that upon stimulation the levels of active RhoA increase in the membrane fractions. In addition, the first Raichu-Rho FRET data from the Matsuda-group obtained during cell division, showed a cell-type specific activation of RhoA all around the plasma membrane and at the cleavage furrow.[35, 36]

It was however difficult for us to imagine how RhoA, which is involved in so many, most rapid, intercellular processes, might fulfill all these functions when activated at only one place. One option is that RhoA after activation at the membrane travels to other locations, like SFs. Considering the tight spatio-temporal regulation of Rho GTPases it is also possible that RhoA is activated at these locations itself. To investigate this we used ECs with a lower Raichu-RhoA probe expression. This allowed us to obtain images with more structural details in the cell body. We showed that upon thrombin stimulation of confluent EC monolayers RhoA was rapidly (<30 sec) activated at cytosolic fiber-like structures, where it remained active during remodeling/contraction of the fiber.

Continued RhoA activity thus seems needed to drive the SF-mediated cell contraction. In agreement with this, in vascular smooth muscle cells of isolated mouse arteries it was currently shown by a FRET-based biosensor for calcium/calmodulin (Ca^{2+}/CaM)-dependent myosin light chain kinase (MLCK) activation that force development as well as force maintenance is Rho/Rho kinase dependent.[37, 38]

Combined, these and other literature data indicated that upon thrombin stimulation of EC monolayers RhoA is transiently activated at fin-like ruffles at the membrane and at cytosolic SFs, where the activity of RhoA and Rho

kinase are required for force development and force maintenance during SF contraction.

Finally, RhoA activity was located at finger-like structures around interendothelial gaps prior to cell separation. The RhoA activity at this location resembles both the RhoA activity at the cleavage furrow during cell separation[35, 36] and the tail retraction of ECs during migration, which was associated with increased Rho-mediated force development.[39]

Van Nieuw Amerongen *et al*.[40] reported that in intact vessels thrombin rapidly induces permeability. Interestingly, high magnification imaging indicated that *in situ* thrombin-induced SF-mediated ECs rounding did not lead to large interendothelial gap formation. One reason may be that *in vivo* thrombin is rapidly inactivated, thereby possibly shortening the EC exposure to thrombin. However, *in vitro* data shows that although the activation-induced internalization of the thrombin receptor causes rapid desensitization,[41-43] receptor signaling goes on until the receptor gets degraded. Another explanation may be the stiffness or lack of stiffness of the cell matrix in vessels as compared to the *in vitro* culture. Due to a higher flexibility *in vivo*, the cell-to-matrix interactions during stimulation of the cells may be more flexible and thus less vulnerable to disruption, thereby reducing gap formation.

To answer these and other questions it would thus be interesting to know whether the here reported RhoA activities play a role *in vivo*. Unfortunately, unlike the FRET-based biosensor for Ca^{2+}/CaM-dependent MLCK activation, where binding of Ca^{2+}/CaM induces an overall increase in MLCK activity in the cell, and thus an overall change in the FRET signal,[37, 38] RhoA activity is local. As a result of this, combined with a bad signal-to-noise ratio *in vivo*, RhoA-FRET analysis with similarly high localization specificity as shown in this thesis is not (yet) possible. As a compromise, the Rho-GTP binding domain (RBD) of rhotekin proteins tagged with GST (see http://www.cytoskeleton.com/, #RT01) was used for localizing active RhoA *in vivo*. Our preliminary data obtained from ECs of small venules of the rat after *in vivo* administration of the thrombin receptor activating peptide (TRAP) supports our *in vitro* findings that RhoA activity is transiently increased and initially located at the cell margins and is later involved in SF-mediated contraction. At least these data indicate that our findings are relevant *in vivo*. Whether there is a protective mechanism upon stimulation of ECs with vasoactive agents requires further investigation.

Thrombin-mediated permeability induces β-catenin-regulated gene expression

Finally, we investigated whether thrombin-activated RhoA/Rho kinase mediated interendothelial gap formation and whether it was involved in altered gene expression induced by the junctional proteins β-catenin and p120ctn. Thrombin indeed caused the relocalization of both catenins from the membrane to the cytosol, which could be prevented by pre-incubation of the cells with the Rho kinase inhibitor Y-27632. Interestingly, within the first 4 minutes of thrombin stimulation we saw a non-significant, but reproducible increase of β-catenin in the membrane while there was a decrease in the cytosol. In combination with our finding that in the same time-period thrombin-induced RhoA activity is located at small fin-like ruffles at the plasma membrane, we hypothesize that the rapid formation of small fin-like ruffles upon thrombin stimulation is an initial response of the ECs to counteract barrier disruption. In line with this, Johnson *et al*.[44] reported that the recruitment and turnover of β-catenin to the membrane ruffles is faster then to the AJs.

In addition, thrombin induced an inhibitory GSK3β phosphorylation, which prevents the phosphorylation of cytosolic β-catenin and subsequently its degradation by proteasomes. Thrombin further mediated a nuclear localization of β-catenin and p120ctn, resulting in the activation of both the TCF-based β-catenin reporter (Topflash) and the β-catenin inducible genes.

The Rho-mediated interendothelial gap formation induced both β-catenin and p120ctn relocation from the membrane to the cytosol and further to the nucleus. Interestingly, only part of the β-catenin-inducible genes we next investigated where enhanced during thrombin-induced permeability. The enhanced genes included cyclin-D1, c-myc, Cox2 and others. Evidence shows that several of these genes, e.g. cyclin-D1 and c-myc, have Kaiso binding sites and that like β-catenin, p120ctn is needed for their expression.[45, 46] Combined, these data indicate that p120ctn/Kaiso signaling regulates a subset of β-catenin target genes, for example those directly involved in specific biological processes such as cell proliferation and migration, two important processes in angiogenesis.

Furthermore we showed that thrombin-induced nuclear β-catenin also activated the TCF/LEF-based reporter Topflash. This may be important since it is now understood that β-catenin also interacts with other transcription factors like HIF-1,[47] c-Jun[48] and FOXO.[49] The shift in co-transcription factor binding of β-catenin was shown under conditions of

increased ROS production, which is associated with vascular development and disease (like vascular leakage, angiogenesis, diabetes, hypertension and atherosclerosis).[50] The diversity in co-transcription factor and the presence or absence of gene-repressors largely explains why under certain conditions only specific subsets of β-catenin-inducible genes are unregulated.

It is of interest to know whether thrombin-mediated permeability indeed leads to β-catenin-driven cell migration and angiogenesis. Although no data is available about permeability-induced β-catenin activity *in vivo*, β-catenin is known to drive VEGF-mediated cell proliferation and mobilization.[51] Using *in vivo* β-catenin-GFP and β-catenin-LacZ reporter models has demonstrated a high transcriptional activity of β-catenin in ECs during vasculogenesis and angiogenesis which decreased during vessel maturation.[52, 53] We would thus like to use these models to investigate the role of β-catenin in permeability-induced vascular adaptations.

In addition, it is urgent to determine the clinical relevancy of β-catenin-mediated gene expression in human vascular diseases in ECs. Although nuclear β-catenin signaling has been associated with diseased tissues,[54,55] there is no data that actually shows nuclear β-catenin in the ECs. Furthermore, determining under which pathological conditions β-catenin is present in the nucleus in combination with the subsequent effect on gene expression may help to further understand the complicated nuclear β-catenin signaling and its contribution to disease.

Concluding remarks

To summarize, the studies presented here have lead to a better understanding of the spatio-temporal regulation of RhoA/Rho kinase activities in the regulation of endothelial permeability. Our *in vitro*, *in situ* and *in vivo* data indicate a dual role for RhoA and Rho kinase in endothelial barrier regulation. Under non-stimulated conditions RhoA activity occurs at membrane ruffles. This may activate mDia. RhoA activity prior to gap formation however, activates Rho kinase, most likely ROCK-I, which via recycling of VE-cadherin to the plasma membrane may stimulate AJ formation and enforces endothelial barrier integrity. In addition, thrombin-induced RhoA and Rho kinase activation at SFs or other actin filaments mediated a mainly ROCK-II-dependent permeability. Together with AJ protein phosphorylation, cell contraction facilitates AJ disassembly, interendothelial gap formation and endothelial permeability.

The AJ disassembly is accompanied by the liberation of β-catenin and p120ctn from the membrane to the cytosol. Here, β-catenin degradation is inhibited by thrombin resulting in an increased nuclear localization of β-catenin. Prolonged nuclear localization of β-catenin and p120ctn next enhances the expression of genes that may be involved in cell proliferation and migration. β-Catenin may thus drive permeability-induced angiogenesis, which is currently under investigation.

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