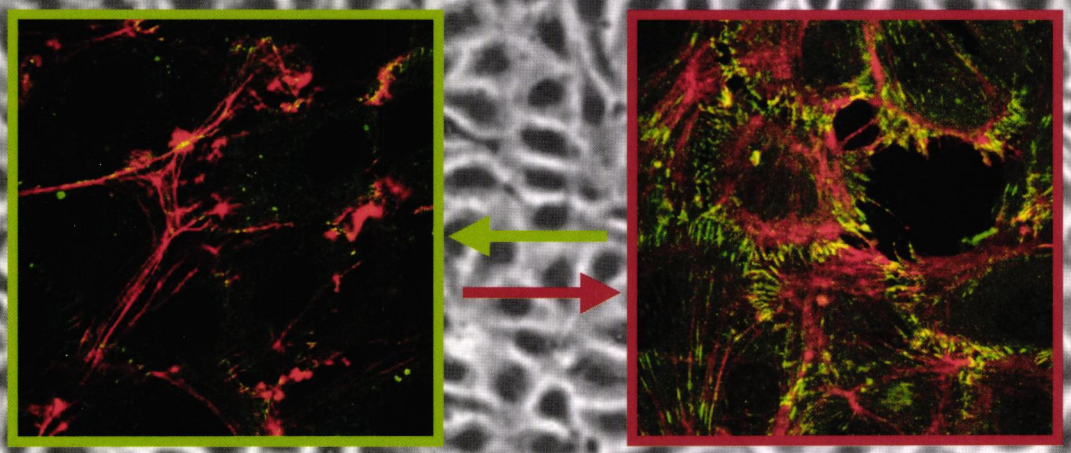
Regulation of the human endothelial barrier function

A study on the mechanisms and modulation of prolonged endothelial hyperpermeability



Geerten van Nieuw Amerongen

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Regulation of the human endothelial barrier function
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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
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De Boelelaan 1105

door

Gerard Peter van Nieuw Amerongen

geboren te Breukelen

Promotor: prof.dr. V.W.M. van Hinsbergh

De grootste meesters en doctores zullen in de
hemel nog minder dan domme leerlingen zijn.
(Theodorus van der Groe 1705-1784)

Aan mijn ouders
Voor Karin en onze kinderen

Content

1

GENERAL INTRODUCTION

Regulation of the permeability of human endothelial cell monolayers (In part adapted from [1])

1.1. Introduction

The endothelium, which forms the inner lining of all blood vessels, separates the blood from the interstitial tissue and is the main physical barrier for blood constituents in their extravasation. The endothelium does not form a passive barrier but actively and selectively regulates the efflux of blood fluid and macromolecules from the blood to the surrounding tissues. Under pathological conditions vascular permeability can increase. Vasoactive agents can activate the endothelium and within a few seconds completely change the permeability characteristics of the endothelium of postcapillary venules, which increases the extravasation of plasma proteins. Vascular leakage may be desirable for recruiting plasma proteins, such as complement factors during infection. However, the formation of edema may result in damage of the tissue and a decrease in the function of (vital) organs. It can be life-threatening, when it occurs in the heart or lungs or when it causes hypovolemic shock. Changes in vascular permeability are associated with a large variety of diseases including cancer, atherosclerosis, diabetes, rheumatoid arthritis and several pulmonary diseases.

This thesis deals with signal transduction pathways, that could play a role in prolonged types of endothelial barrier dysfunction. Various aspects of the endothelial barrier function have been reviewed during the past few years[1-8]; an excellent in-depth-overview of microvascular permeability is given by Michel and Curry.[9] In the current chapter some basal characteristics of the endothelial barrier will be described. The focus of the chapter will be on the various mechanisms, known to be involved in enhanced endothelial permeability. From this the reason and the aim of the studies presented in this thesis will be formulated. The chapter will be concluded with an outline of this thesis.

1.2. Nature of the changes in endothelial barrier function

Among the many functions of the endothelium (reviewed in references [10] and [11]), which include regulation of vascular tone, active provision of a nonthrombogenic surface, and control of neovascularization, its property of forming a physical barrier between blood and interstitial fluid takes a prominent place. Only a few exceptions to this rule exist, notably endothelia in the liver, the adrenal gland and the bone marrow, which have rather large pores. In many different diseases the endothelial barrier does not function properly. This makes it unlikely that one single mechanism could explain the changes in the endothelium which cause vascular leakage. Indeed evidence exists for multiple types of vascular leakage depending on the area of the vascular bed affected, the stimulating vasoactive substances involved etc.

Under healthy conditions macromolecules can cross the endothelial barrier essentially in three ways (see Figure 1): (a) between the cells, through cell junctions, which is called the paracellular pathway for extravasation corresponding to situation 3 in Figure 1; (b) through the endothelial cells, via pores which are either diaphragms or fused vesicles corresponding to situation 5; and (c) transcellularly, via shuttling vesicles and specific receptors corresponding to situation 4 and 6 respectively. Gasses freely diffuse through the endothelium (situation 1) and small lipophilic molecules diffuse through the membrane lipid bilayers (situation 2).

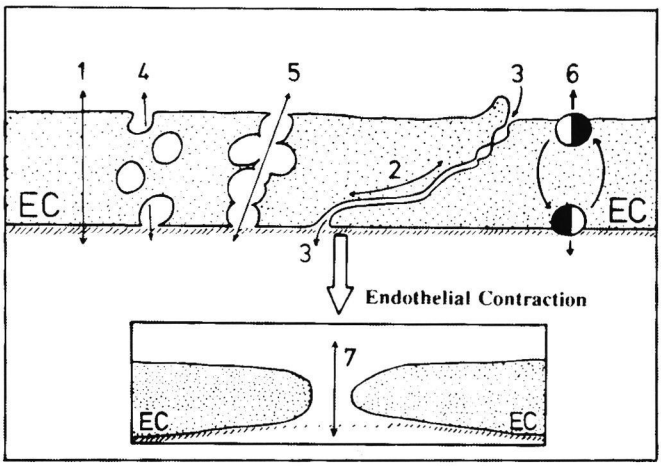

Figure 1. Schematic representation of various exchange pathways which may be involved in the permeation of various nutrients, fluid and macromolecules across the endothelium. 1. diffusion through the cell membranes and the cytoplasm; 2. lateral diffusion of small lipophilic molecules through the lipid bilayers of the cellular junctions; 3. extracellular passage through the narrow slits of endothelial junctions; 4. vesicular exchange; 5. transendothelial channels which consist of fused vesicles; 6. carrier-mediated exchange and receptor-mediated transport. The figure depicts the junctional area between two endothelial cells (EC). Inset: gaps are observed between endothelial cells in activated postcapillary venules.[108] Reproduced with permission from reference [109].

The most prevalent form of dysfunction of the endothelial barrier, resulting in vascular leakage, occurs in the postcapillary venules during acute inflammation.[12; 13] This type of vascular leakage is associated with the development of gaps between neighbouring ECs[12], and can be mimicked by the application of inflammatory mediators like histamine, substance P and Platelet Activating Factor (PAF) to healthy microvessels, in particular the postcapillary venules (see inset of Figure 1, situation 7). The formation of these small intercellular gaps is

caused by a contraction of the ECs. Gap formation is also the underlying mechanism of capillary leakage syndrome.

In the endothelium of large arteries and veins focal leaky spots are sometimes encountered. They are found after exposure of the vessel to injurious conditions, such as occurs in hypercholesteremia[14] and atherosclerosis.[15] The formation of gaps may also contribute to this type of barrier dysfunction. Evidence was provided by many studies (reviewed in [16]), that during the development of atherosclerosis, the permeability of the aortic wall for LDL markedly increases. This increased vascular permeability may contribute to the development of atherosclerosis.

The capillary microvessels become leaky in angiogenesis.[17; 18] The angiogenic factor Vascular Endothelial Growth Factor (VEGF), initially identified as Vascular Permeability Factor (VPF)[19], induces both the formation of intercellular gaps and intracellular clusters of connected vesicular structures, the so-called vesicular vacuolar organelles, and pores within ECs.[20; 21] The relative contribution of these phenomena to the *in vivo* effects of VEGF, however, is still being debated. Comparison of the effects of VEGF with that of histamine revealed that the formation of vesicular structures is specific to VEGF.[20] VEGF affects postcapillary venules, venules and capillaries.[20; 22] VEGF is also a likely candidate to cause the hyperpermeability of tumor blood vessels.[4] The blood vessels that supply tumors differ from those supplying normal tissues and do not correspond closely to the arterioles, capillaries and venules.

So, large arteries, capillaries, poscapillary venules, and tumor vessels, but not large veins are known to develop an impaired barrier function. Remarkably, these areas in the vascular tree correspond to areas that can develop contractile F-actin filaments or cables. *In vivo* these F-actin cables, of which stress fibers (SFs) are a prominent group, occur mainly in large arteries[23; 24], to a lesser extent in the entire microcirculation[25; 26], but are largely absent from the venous system.[24; 27; 28] Many studies have shown that SFs develop during endothelial cell adaptation to unfavorable, or pathological situations including wound healing, atherosclerosis and hypertension.[23; 27; 29-32] This suggests that EC F-actin cytoskeleton plays an essential role in the regulation of endothelial permeability.

In the present study an investigation was made predominantly of changes in endothelial permeability as a consequence of endothelial contraction, the most prevalent type of vascular leakage.

1.3. Endothelial gap formation

A striking feature of the acute inflammatory response is the massive leakage of macromolecules, particularly in the postcapillary venules.[12; 13] Vasoactive agents can increase the permeability of endothelial monolayers by increasing actin-nonmuscle myosin interaction and by inducing the disintegration of endothelial cell-to-cell interactions (see Figure 2). On the basis of morphological observations Majno *et al.*[12; 33] anticipated in the late 1960s that endothelial contraction caused this increase in permeability. However, only in 1990 did Wysolmerski and Lagunoff[34; 35] and Schnittler *et al.*[36], using permeabilized endothelial cells *in vitro*, demonstrate that contraction occurs.

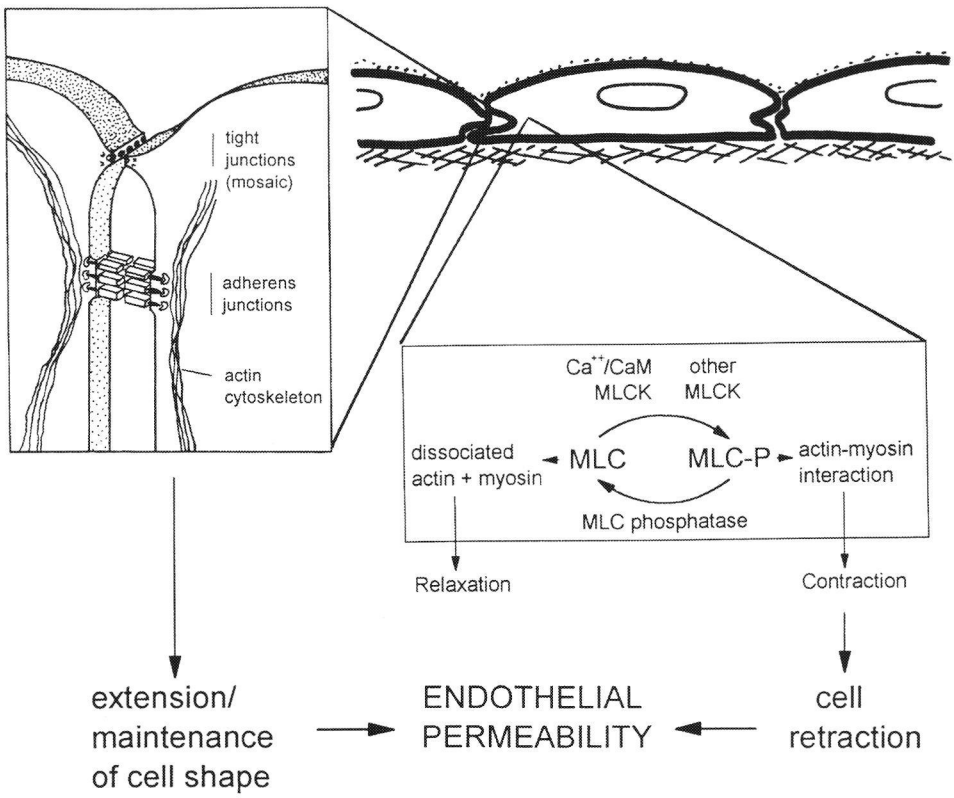

Figure 2. Vasoactive agents can increase the permeability of endothelial monolayers increasing actin-nonmuscle myosin interactions regulated by the phosphorylation of the MLC (right) and by inducing the transient disintegration of cell-to-cell adherens junctions (left). CaM indicates calmodulin. Reproduced with permission from reference.[2]

The contraction should not be seen as a general shrinking of the endothelial cell, but rather as a process that is localized in the periphery of the cell and causes the formation of small gaps between the cells without tearing the cells apart. These small gaps were beautifully visualized by Baluk *et al.*[37; 38] using an *in vivo* silver-staining technique. This process required interaction of actin and nonmuscle myosin and the presence of adenosine triphosphate ATP and Ca^{2+}-ions the calcium-binding protein calmodulin (CaM) and myosin light chain (MLC) kinase. The mechanism of contraction in ECs is comparable to that in smooth muscle cells (SMCs), although many of the proteins involved in the contraction process and its regulation are different in the two cell types (see Garcia *et al.* for a review [39]). As in SMC contraction, the interaction between actin and nonmuscle myosin in ECs is regulated by the phosphorylation status of MLC.[35; 39-41] The activated MLC kinase can phosphorylate, by hydrolysis of ATP to ADP, the 20 kDa MLC, which is located at the myosin head.[42; 43] Phosphorylation of the MLC promotes binding of myosin to actin filaments and initiates the actomyosin complex ATPase activity.[44] The chemical energy liberated after ATP hydrolysis is the driving force for the conformational change of the crossbridge between myosin and actin filaments, leading to filament movement. When ECs *in vitro* are stimulated by histamine or thrombin, which induces a transient or prolonged increase in permeability, respectively, mono- or diphosphorylated MLCs are generated. Within 1 or 2 minutes phosphorylation reaches a maximum, which follows an increase in $[Ca^{2+}]_i$.

The induced increase in permeability can be at least partially inhibited by intracellular Ca^{2+} chelators or inhibitors of calmodulin and MLC kinase.[35; 36; 39; 40; 45] Therefore, it is generally believed that the Ca^{2+}/calmodulin-dependent PK 1 (the classic MLC kinase) plays a central role in the onset of endothelial gap formation after exposure to vasoactive agents, although other MLC kinases may be involved. The identity of these MLC kinases is discussed in Chapter 2. At this point it is sufficient to remark that evidence exists that EC MLC kinase differs from MLC kinase from SMC.[46]

From these experiments it can be learned that vasoactive agents can increase endothelial permeability by the formation of small gaps between neighbouring cells. These gaps are formed by a contraction process at the margins of the cells. This contraction has many characteristics in common with SMC contraction and depends on a Ca^{2+}/CaM-dependent activation of the MLC kinase.

1.4. Intracellular signaling pathways involved in endothelial cell contraction

Additional information obtained from *in vitro* experiments showed that regulation of endothelial barrier function is more complicated. We will describe signaling events in ECs

resulting in barrier dysfunction using histamine and thrombin as examples as histamine and thrombin are exemples of vasoactive compounds able to induce a transient or a prolonged endothelial hyperpermeability response respectively. Histamine is largely involved in the inflammatory reactions and was one of the first vasoactive agents known to induce vascular leakage and endothelial gap formation *in vivo*.[12; 33] High affinity binding sites for histamine were identified in venules.[47] Thrombin has been used for many years as a standard stimulatory agent to induce a prolonged endothelial barrier dysfunction *in vitro*. A number of reports described *in vivo* effects of thrombin in vascular leakage.[48-52] Intravenous infusions of thrombin cause increased pulmonary microvascular permeability to proteins resulting in pulmonary edema in animals. Signal transduction pathways used by histamine[53-55] and thrombin[56] receptors have been characterized extensively. We will restrict ourselves by summarizing the knowledge regarding pathways known to play a role in endothelial permeability.

Thrombin and histamine share the ability to activate a G-protein-coupled phospholipase C leading to inositol trisphosphate (IP3)-mediated increases in $[Ca^{2+}]_i$ and rapid diacylglycerol (DAG)-induced activation of PKC. As indicated above increased $[Ca^{2+}]_i$ appears to be critical for agonist-mediated EC contraction. The rise in $[Ca^{2+}]_i$ induced by either histamine or thrombin in ECs is very comparable, both in magnitude and duration.[57] Both histamine and thrombin at a maximal effective concentration induce a very rapid and transient increase in $[Ca^{2+}]_i$ in ECs, that returns to basal levels within 5 minutes. In close agreement with these data, it has been observed *in vivo* that a transient increase in $[Ca^{2+}]_i$ parallels an increase in endothelial permeability after exposure of frog mesenteric microvessels to histamine.[58] In this case the endothelial barrier recovers within several minutes. Other inflammatory mediators like substance P, ATP, serotonin, and bradykinin, were also found to induce a transient increase in vascular permeability, similar to the initial phase of inflammation.[9; 37; 58-61] However, stimulation of endothelial monolayers *in vitro* with thrombin induces a prolonged increase in endothelial permeability, that extends far beyond the increase in $[Ca^{2+}]_i$. This suggests that in addition to a rise in $[Ca^{2+}]_i$ prolonged increases in endothelial permeability require other or at least additional activation or sensitization steps than the rise in $[Ca^{2+}]_i$. This is supported by the finding that the thrombin-induced increase in endothelial permeability is inhibited only partly by chelators of $[Ca^{2+}]_i$.[45; 62] Furthermore, stimulation with thrombin-receptor activating peptide (TRAP) induced a similar rise in $[Ca^{2+}]_i$ compared to thrombin, but did not stimulate a prolonged increase in endothelial permeability, indicating that elevation of $[Ca^{2+}]_i$ is not sufficient for prolonged hyperpermeability.[63]

Measurements of isometric tension in ECs demonstrated that the thrombin-increased isometric tension was accompanied by MLC phosphorylation to a considerable extent, whereas histamine had a much smaller effect.[40; 41] The effects of thrombin were inhibited by cytochalasin D and an MLC kinase inhibitor, indicating that actin and MLC phosphorylation play a role in thrombin-induced isometric contraction. These data strongly suggest that an increase in actin-myosin-dependent isometric tension contributes to the prolonged increase in endothelial permeability.

The involvement of isometric contraction does not exclude the simultaneous involvement of other mechanisms. In particular, signal transduction via protein kinase C (PKC) and protein tyrosine kinases (PTKs) has been implicated in the regulation of endothelial permeability. Several authors have reported that activation of PKC contributes to the thrombin-induced increase in permeability of bovine ECs.[64-66] In human ECs the contribution of PKC could not be demonstrated unequivocally. Yamada *et al.*[67; 68] showed that activation of PKC caused a decrease in endothelial permeability, whereas Bussolino et *al.*[69] found an increase in permeability by PKC activation. The finding that thrombin and histamine induce a comparable rise in $[Ca^{2+}]_i$ makes a major contribution of activation of Ca^{2+}-dependent PKC to prolonged increases in permeability unlikely.

Rabiet *et al.*[70] reported that thrombin disrupted the VE-cadherin-catenin complex in adherens junctions. The disruption of adherens junctions could be prevented either by inhibition of PKC or PTK. This suggests that in addition to the contraction mechanism, that involved actin-nonmuscle myosin interaction, disintegration of adherens junctions contributes to the increased permeability. The disintegration of adherens junctions is reversible after several hours. These data suggest that disintegration of adherens junctions between cells and also the possible loss of focal contacts between the cell and its matrix may contribute largely to the prolonged leakage induced by thrombin *in vitro*.

Studies published on the microcirculation have demonstrated a short-lasting transient leakage in postcapillary venules after stimulation with a vasoactive agent. These observations seem to give little support to the concept of prolonged leakage sites. However, these observations were made in healthy tissues and with a single stimulus. It is indeed conceivable that disintegration of cell-cell contacts and prolonged leakage occurs, particularly in areas of inflammation where leukocytes adhere to ECs and act on them by producing factors like PAF and TNF, that could sustain the vascular leakage. In many cases, only the initial phase can be inhibited by antihistamines.[71] Adherent leukocytes induce cytoskeletal changes in ECs comparable to thrombin and activate MLCK.[72-74] Therefore, it is

desirable to elucidate the mechanisms involved and their regulation, because additional approaches may be required to treat such cases of vascular leakage.

1.5. Stabilization of the endothelial barrier

As outlined above contractile forces induced by vasoactive agents result in the formation of gaps between endothelial cells. These contractile forces generate a centripetal tension. Measurements of isometric tension showed that a basal tension exists, that does not result in a dysfunction of the endothelial barrier. So, opposing centrifugal forces must exist that counteract the contractile forces. The concept has been developed that a dynamic equilibrium of opposing contractile and adhesive forces regulates endothelial barrier function.[39; 40]

The adhesive forces consist of tethering forces exerted by cell-cell and cell-matrix interactions. The most important structures that are responsible for the cell-cell and cell-matrix interactions are the adherens junctions and focal adhesions respectively.

In the endothelium at least four types of junctions between neighbouring ECs have been described. These are: tight junctions, adherens junctions, gap junctions and syndesmos.[75-80] Of these junctions the tight and adherens junctions are important for the barrier properties of the endothelium.

The tight junctions or the zonulae occludens separate the blood compartment from the interstitium.[81] In the idealized classic view of interendothelial junctions the tight junctions and adherens junctions are spatially separated. Recent evidence, however, indicated that the tight junctional protein claudin-5 exactly colocalized with the adherens junctional protein VE-cadherin.[82] Tight junctions appear as a network of linear fibrils circumscribing the cells and can be regarded as a kind of isolator in brain endothelium. In other endothelia tight junctions do not form a belt of multiple ridges but probably form a kind of mosaic structures as previously depicted by Bundgaard.[83] Tight junctions do not form a continuous seal, but contain pores that can be regulated to achieve selective molecular sieving.[81; 84] Tight junctions probably do not contribute to adhesive forces between the cells.

Adherens junctions are cellular membrane contact sites formed by cadherins, which mediate the physical attachment between cell membrane and the intracellular F-actin cytoskeleton. The endothelium expresses a specific cadherin, cadherin-5 or VE-cadherin, which is strictly located at intercellular junctions of essentially all types of endothelia. VE-cadherin is complexed with several catenins, that are linked via α-actinin to actin filaments. The organization of these complexes suggests that adherens junctions play an essential role

in the regulation of gap formation and endothelial barrier function. Adherens junctions are not only involved passively in the contraction process, as the site where the cytoskeleton can exert tension on the cell membrane and pull the cells away from each other, but the organization of the adherens junction is actively regulated by vasoactive agents. Histamine and thrombin have been shown to induce phosphorylation (on tyrosine residues) of the adherens junctional proteins VE-cadherin and the catenins, which results in a redistribution of these proteins.[70; 85; 86]

Several signaling molecules have been shown to improve endothelial barrier function. Cyclic AMP (cAMP), generated by activation of adenylate cyclase, is one of these signaling molecules that stabilizes endothelial junctions, inhibits MLC kinase and prevents agonist-induced endothelial gap formation.[87-89] ß-adrenergics agents and serotonin increase cAMP levels in ECs and were shown to improve endothelial barrier function. Therapeutic use of these agents, however, is still limited, because desensitization processes occur that shorten the time these compounds are effective in reducing vascular leakage.[90; 91]

Another signaling molecule that was shown to improve barrier properties *in vitro* is cGMP. The effects of cGMP on *in vivo* barrier properties, however, are controversial and depended on many factors, such as the vascular bed involved and the condition of the endothelium. cGMP is generated by a Ca^{2+}/nitric oxide (NO)-dependent pathway or by cGMP-increasing agonists.[92] Several mechanisms have been identified by which cGMP could improve endothelial barrier function *in vitro*. The first is a feedback mechanism by activating cGMP-dependent protein kinases.[45; 93] In ECs of large arteries and veins, but not in those of the umbilical veins and kidney glomeruli where cGMP-dependent protein kinase I is absent, activation of cGMP dependent kinase I reduces the agonist-induced rise in $[Ca^{2+}]_i$ responsible for the increase in permeability. The second mechanism by which cGMP could act is the elevation of intracellular cAMP levels, by inhibition of a cAMP-degrading phosphodiesterase. This mechanism accounts for the reduction in endothelial permeability by cGMP in ECs of the umbilical vein.[45] No therapeutic application of NO/cGMP elevating agents with regard to the improvement of endothelial barrier function is currently available.

1.6. Relationship between endothelial permeability and angiogenesis

A striking feature of microvascular leakage is that it often accompanies the formation of new blood vessels. In inflammation a first phase can be distinguished from a second phase. In the first phase blood vessels dilate, become hyperpermeable and diapedesis of leukocytes occurs. In the second phase more structural changes in the microvessels occur. There is remodeling of existing vessels and formation of new vessels from existing ones.[94; 95]

Another example is activation of the endothelium by VEGF, initially manifested by an increase in endothelial permeability and edema formation[19; 96] , followed by the formation of new blood vessels.[97] These findings suggest a relationship between increased endothelial permeability and angiogenesis. Indeed, it was found that microvascular hyperpermeability plays a mechanistic role in angiogenesis. The most extensive evidence for an association between microvascular hyperpermeability and angiogenesis has come from studies of solid tumors[21; 96] Tumor blood vessels are hyperpermeable.[4] Plasma proteins extravasate from the blood vessels that supply tumors and form a new provisional extravascular fibrin matrix that permits and indeed favors the inward migration of endothelial cells (reviewed in [21]). This mechanism is probably not specific to formation of tumor blood vessels as many similarities exist between tumor stroma generation and wound healing.[95; 98] Other conditions of extravascular fibrin deposition include corpus luteum formation, psoriasis, contact allergy, and rheumatoid arthritis.[99-102] All these conditions are associated with enhanced angiogenesis, and support the idea that enhanced plasma protein extravasation favors angiogenesis.

1.7. Determination of endothelial barrier function in an *in vitro* model

Because observations in endothelial monolayers *in vitro* can be directly compared with biochemical data from cell extracts or permeabilized cells, such monolayers provide a flexible tool for answering questions regarding the regulation of endothelial permeability. An *in vitro* model to evaluate endothelial barrier function was developed in the past in our laboratory and extensively characterized.[103] This model consists of a porous membrane on which endothelial cells, both of macro- and microvascular origin[45] are cultured to tight confluent monolayers (see Figure 3).

In the studies of this thesis only endothelial cells from human origin were used. All of the cells grown on the porous filters are connected by tight junctions (as seen by transmission electron microscopy). The monolayers have a transendothelial electrical resistance (TEER) in the physiological range. Their barrier properties depend on Ca^{2+}-ions and the presence of albumin. Small molecules pass considerably faster (> 100-fold) than large molecules. Very large molecules, such as very-low-density lipoprotein (VLDL), do not pass through the monolayer, although the permeability is an order of magnitude higher than that of the continuous endothelium *in vivo*.

Barrier function can be assessed either by measuring the TEER or passage of a tracer molecule through the endothelial monolayers. TEER can be measured in a special chamber, as was developed at our institute previously.[103] To determine permeability of

endothelial monolayers passage experiments tracer molecules can be chosen in a large variety of molecular weights or Stokes radii and include ^{22}Na-ions, radiolabeled sugars, fluorescent dextrans, horse radish peroxidase (HRP), and radiolabeled albumin or lipoprotein particles depending on the purpose of the study.[103-105] In this study HRP was used as a tracer molecule in most cases. HRP passes the endothelium *in vivo* via the paracellular route.[106] For a more detailed description of determination of endothelial integrity the interested reader is referred to a book chapter in which further technical data were given.[107]

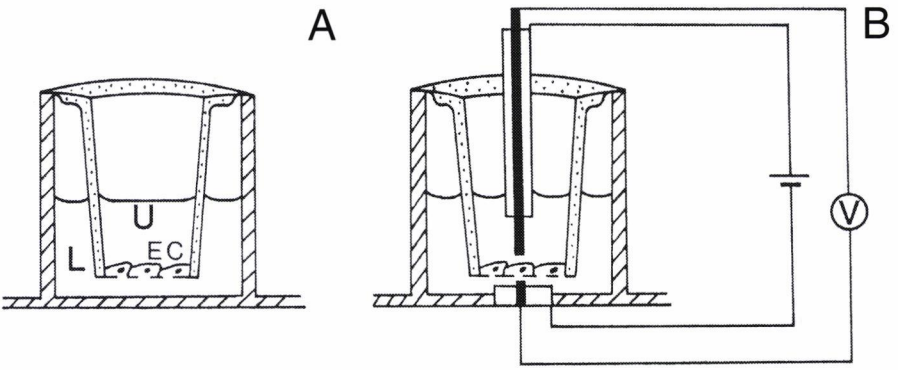

Figure 3. Schematic view of the assay system for measuring the diffusion of macromolecules through monolayers of human endothelial cells. Endothelial cells are seeded in a high density and cultured for 4-7 days to form a tight endothelial cell monolayer. During the experiments a known amount of tracer molecules is added to the upper compartment (U); at several time-points a sample is taken from the lower compartment (L). Experimental details are given in reference.[107] B: The trans-endothelial electrical resistance (TEER) is measured in a special chamber. An alternating current passes the monolayer by two source electrodes. The two other electrodes detect the potential difference across the monolayer. Reproduced with permission from reference.[103]

1.8. Aim and outline of this study

The major aim of the present study was to investigate which mechanisms together with a rise in $[Ca^{2+}]_i$ and the Ca^{2+}/CaM-dependent activation of the MLC kinase are involved in prolonged types of leakage in order to find new therapeutic targets for treatment of vascular leakage.

During our study we became aware of the contribution of the Rho family of small GTPases to regulation of endothelial permeability. Chapter 2 will give an overview of current knowledge regarding the role, function and characteristics of Ras-related small GTPases of

the Rho family in the vascular system, as it has become clear in the last 2 years that these small GTPases are involved in many vessel pathologies and as they were one of the main subjects of investigation of the present study.

In chapter 3 a dissection was made between transient and prolonged increases in endothelial permeability effects in an *in vitro* model. Histamine was chosen to study as a vasoactive agent inducing transient barrier failure, and thrombin as a mediator inducing prolonged increases in permeability. The signal transduction pathways involved in transient and prolonged barrier dysfunction were investigated. Under well-defined conditions we were able to simulate the effects histamine has on permeability of healthy microvessels *in vivo* in an *in vitro* model, allowing us to study signaling pathways involved in transient endothelial barrier failure. The transient effects of histamine were shown to be fully dependent on the rise in $[Ca^{2+}]_i$. On the other hand the prolonged increase in permeability thrombin induced in this *in vitro* model was dependent on activation of protein tyrosine kinases and RhoA –a member of the Rho family of small GTPases- in addition to a rise in $[Ca^{2+}]_i$. The involvement of protein kinase C was evaluated. The thrombin-induced endothelial permeability was accompanied by a dramatic reorganization of the F-actin cytoskeleton, the formation of F-actin stress fibers and focal adhesions.

In chapter 4 the role of RhoA in increased endothelial permeability is described in more detail, using the specific Rho activator lysophosphatidic acid (LPA). In the absence of any detectable rise in $[Ca^{2+}]_i$ activation of RhoA by LPA results in an increase in endothelial permeability. Disturbance of barrier function induced by LPA is not as strong as in the case of thrombin, where both Ca^{2+}-ions and RhoA are involved. Furthermore, evidence is provided that effects of RhoA are mediated by one of its targets, the Rho-dependent kinase or Rho kinase. In addition to adding new information regarding intracellular signaling mechanisms involved in endothelial barrier function, these findings identify LPA as a new possible mediator, which may induce an endothelial barrier dysfunction.

In chapter 5 direct evidence is provided that thrombin activates RhoA in ECs using a newly developed assay for RhoA activation. Under the same conditions the related GTPase Rac was not activated. Rho kinase was shown to be involved in the thrombin-enhanced endothelial barrier dysfunction. The role of PTK in activation of RhoA and endothelial permeability was investigated. Evidence was provided that PTK do not to act upstream of Rho activation and probably not downstream of Rho kinase, but act in parallel to activation of Rho kinase.

Based on the recent finding that statins, drugs frequently used in lipid-lowering therapy, can inhibit Rho function and the new insights that activation of RhoA could play a

role in endothelial barrier dysfunction we hypothesized that statins could improve a disturbed endothelial barrier function. In chapter 6 we tested this hypothesis using simvastatin, both in our *in vitro* model of thrombin-induced endothelial barrier dysfunction and in an *ex vivo* assay for endothelial integrity in atherosclerotic rabbit aortas.

In inflammation the initial increase in vascular permeability is often followed by the formation of new blood vessels. The same is observed when the endothelium is activated by VEGF. VEGF is a well-known inducer of *in vivo* vascular leakage, which is followed by angiogenesis. In chapter 7 we investigated whether the same RhoA/Rho kinase signaling pathways as involved in prolonged increases in endothelial permeability could contribute to prepare ECs for the formation of new blood vessels. We investigated whether Rho kinase is involved in the migratory and angiogenis effects of VEGF on endothelial monolayers *in vitro*.

The consequences of the findings of this study for the treatment of vascular leakage are discussed in chapter 8 and directions for future research will be indicated.

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2

CYTOSKELETAL EFFECTS OF RHO-LIKE SMALL GUANINE-BINDING PROTEINS IN THE VASCULAR SYSTEM

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2.1. Introduction

After the discovery of the central role of the Rho family of small or low-molecular-weight GTPases as regulators of the actin cytoskeleton[1;2] in the early 1990s, it was found that these small GTPases were also involved in gene regulation and cell cycle progression. Afterwards, a large body of evidence was obtained of the important functions of Rho GTPases in many processes in the vasculature, as diverse as regulation of (elevated) blood pressure, platelet activation, wound healing or leukocyte extravasation. The aim of the current review is to give an overview of the evidence obtained in which processes Rho GTP-binding proteins (G proteins) are involved in the vascular system, with emphasis on the cytoskeletal effects and just when necessary mention their effects on gene regulation. This in order to learn the general patterns these small GTPases use, to see what determines specificity in each process and how these processes can be modulated pharmacologically. However, we are just at the beginning to understand how these different processes are coordinated and integrated. This review is not aimed to review all possible modulators of GTPase activity, all target molecules of Rho GTPases or to describe their moleculair structure, their involvement in gene regulation, or cell cycle progression, as this is done by many other authors (Rho signaling pathways[3-8], Rho as a mediator of GPCR signaling[9-11], Rho GTPases and the cytoskeleton[12-20], Rho and integrin function[21-24], Rho and gene regulation[25], Rho and development[26]), but to describe current knowledge of the roles of small GTPases in the vasculature.

2.2. General outline of Rho-like small GTPase action

2.2.1 The Rho GTPase family

The Rho proteins belong to the widespread Ras superfamily of small G proteins[27], from which they got their name as Ras homologues. Prototypes of the Rho protein family are Rho, Rac and Cdc42. Rho GTPases are key regulators of the actin cytoskeleton. By their action on the actin cytoskeleton, they play a major role in fundamental processes as cell contraction, cell motility, cell adhesion and cell shape. It is therefore not surprising that knockout mice of Rho GTPases often are not viable[28], as these proteins fullfill many essential functions. The Rac2-/- mouse is the only published Rho GTPase KO mouse thus far, but Rac2 seems to be exceptional as Rac2 expression is restricted to haemapoetic cells.[29] Some of the molecular pathways that connect Rho GTPases to the control of the cytoskeleton have been established now.

With the identification of more members and isoforms, a confusing nomenclature has developed the last years. Members of the Rho protein family can be divided in six different

classes consisting of the following members: Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, and Rac3, which is also known as Rac1B, RhoG), Cdc42 (Cdc42Hs, G25K and TC10), Rnd (RhoE/Rnd3, Rnd1/Rho6, Rnd2/Rho7), RhoD, and TTF.[11;30] In this list RhoE is the same as Rnd3, which will be discussed below, RhoF does not exist, and Cdc42, TC10 and TTF miss the R in their name to identify them as members of the Ras superfamily of proteins.

Rho, Rac and Cdc42 are the three classes of which most is known. Each has its own specific effects on the actin cytoskeleton, likely resulting from the activation of different protein subsets involved in actin polymerization.[27;31] A striking feature of activation of Rho is the formation of cytoplasmic stress fibers (SFs) in cultured cells, that can form SFs, and an increase in actomyosin-based contractility in cells that cannot form SFs (such as neuronal cells).[2] SFs are long cytoskeletal cables or bundles of actin and myosin II/non-muscle myosin filaments, that can contract and exert tension (see below under '*Stress fiber formation*') and are linked to the plasmamembrane at focal adhesions (FA). Rac and Cdc42 regulate peripheral F-actin assemblies. Rac is involved in the formation of membrane ruffles and lamellipodia meshworks[1], whereas Cdc42 induces the formation of radial, unipolar bundles, termed microspikes or filopodia.[32]

All three protein classes also can regulate the assembly of integrin-containing FA complexes and thus regulate cell-matrix interactions and cell adhesion.[22;24] Rho induces the formation of the classical FAs. These integrin-containing complexes are connected to bundles of SFs and are clustered over the basal surface of the cell, maintaining their firm attachment to the underlying substratum. Rac and Cdc42 induce the formation of the smaller focal contact sites at the cell periphery, associated with lamellipodia and filopodia.[33]

Recently, new members of the Rho family of small GTPases, Rnd1, Rnd2 and Rnd3/RhoE, have been identified.[34] Rnd proteins have a close homology to RhoA, but display a very distinct biochemical behaviour. Rnd proteins lack GTPase activity and are constitutively in the active state. Therefore, expression levels of Rnd proteins primarily determine their involvement in signaling. Expression of these proteins in fibroblasts causes cell rounding (Rnd for 'round') and inhibits formation of SFs, lamellipodia and FAs and appears therefore to have an antagonistic effect to Rho and Rac.[35]

2.2.2. Rho GTPase signaling

Rho GTPases can be activated principally by 2 different ways: by soluble factors via heterotrimeric G protein coupled receptors (GPCRs), tyrosine kinase receptors and cytokine receptors[9;11] or by cell adhesion and integrin clustering.[36-39] One example of the former is cellular activation by lysophosphatidic acid (LPA), which was the first agonist identified to activate Rho.[2] LPA is present in serum, and activates Rho via several seven-transmembrane

GPCR.[40-42] Many other GPCR-agonists have been described to activate Rho-family GTPases, including thrombin, endothelin, carbachol, PGE2, bombesin, fMLP, angiotensin, α-adrenergica, sphingolipids (reviewed in[10]). Activity of low-molecular-weight G proteins is not directly regulated by agonist binding to GPCRs, as is the case with the α-subunit of the heterotrimeric G proteins, but is regulated in another way. Similar to other G proteins, low-molecular-weight G proteins, with exception of the Rnd proteins, are molecular switches, which can bind either GDP or GTP, which results in a change in conformation. They are active in their GTP-bound status and inactive in the GDP-bound form. In the GTP-bound form, Rho GTPases interact with and activate their target molecules. The capacity to cycle between 2 conformations enables these molecules to amplify or to temporise upstream signals. Indeed, it was shown recently in neuronal cells, by fishing for GTP-bound RhoA with the Rho-binding domain of Rho kinase, that LPA induced an increase in GTP-bound Rho in a PTK sensitive way via Gα12/13, concomittant with growth cone collapse.[43] Activation of Rho is accompanied by an increase of membrane-associated Rho and decrease of cytosolic Rho, indicative of Rho activation.[44;45]

Activity of small G proteins is under direct control of large set of other regulatory proteins: specific factors that activate GTPases, specific factors that turn them off and finally specific factors that keep them in their inactive state (see Figure 1). For each of these regulating factors several different molecular entities have been identified and this number still is growing. Guanine nucleotide exchange factors (GEFs) enhance or catalyse the exchange of GDP for GTP.[10] The slow intrinsic rate of GTP hydrolysis of the small GTPases is enhanced by GTPase-activating proteins (GAPs), whereas guanine dissociation inhibitors (GDIs) slow the rate of GDP dissociation from the GTPases, thereby locking the G proteins into the inactive state.[46] These GDIs bind to the carboxyl terminus of Rho. In this way, they prevent the translocation of the GTPases from the cytosol -where the major fraction of eg. Rho is present under nonstimulated conditions complexed to RhoGDI- to the plasma membrane, when activated (reviewed in [46]). ERM proteins (ezrin, radixin and moesin) also have been implicated in membrane recruitment. In quiescent fibroblasts Rho, Rac and ERM proteins, but not RhoGDI are enriched in caveolae membranes compared to plasma membranes. Stimulation with growth factors results in a further recruitment of Rho, Rac and ERM proteins.[47] In ECs RhoA colocalizes with ERM proteins.[48]

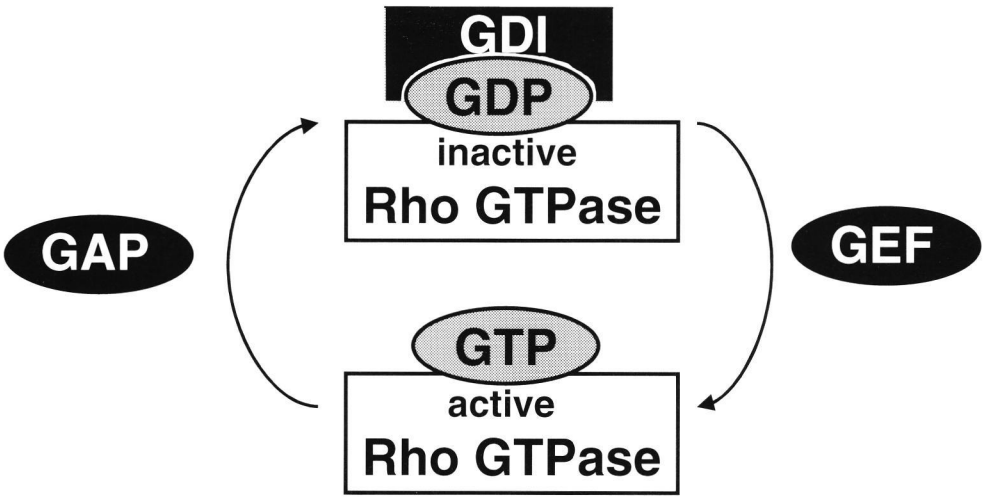

Figure 1. The activation/inactivation cycle of Rho GTPases is a highly regulated process. Guanine dissociation inhibitors (GDI) keep the Rho GTPases in their inactive state by slowing the rate of GDP dissociation from the GTPases. Guanine exchange factors (GEF) enhance the exchange of GDP for GTP, thereby activating the Rho GTPases. The slow intrinsic rate of Rho GTPase-inactivation is enhanced by stimulation of the GTPase activity by GTPase-activating proteins (GAP).

Upstream signaling events, which result in activation of the small GTPases have been unknown for a long time and it is just very recently, that two possible mechanisms were identified by which GPCR stimulation results in activation of small GTPases. The first was the identification of a direct link between Gα and p115RhoGEF.[9;10;49;50] The second is a ligand-independent activation of the EGF receptor acting upstream of Rho.[51]

In fibroblasts Cdc42, Rac and Rho initially were found to act in a hierarchical cascade[32], which also seems to be true in some cases in ECs.[52] Later on, it turned out that this cannot be taken as a general rule, as more recent reports indicate that Rac and Rho also have some mutually antagonistic effects.[33;53;54] This becomes immediately clear as one compares the cell extension promoting effects of Rac and Cdc42 with the cell contraction promoting effects of Rho.

A number of downstream effectors of Rho GTPases has been identified and this number is still growing. Of many of them a function is unknown at the moment. Rho effectors include Rho kinase, which is also known as P160ROCK, exists as 2 isoforms, ROCKI and II or ROKβ and ROKα respectivily[55-58], the myosin binding subunit (MBS) of the myosin phosphatase[59], protein kinase N/PRK1[60;61], rhophilin[61], rhotekin[62], citron[63;64], Kv1.2[65], phospholipase D[66], and Dia.[67] Rac effectors include p21 activated kinases (Pak)[68;69],

p67phox (component of the NADPH oxidase complex), IQGAP1, POR1, p140 Sra-1 and POSH.(reviewed in [70]) Pak, WASP, IQGAP1 and MRCK are identified as effectors of Cdc42.[70]

Thus, Rho GTPases with their three main representatives Rho, Rac and Cdc42 are key regulators of the F-actin cytoskeleton. By their ability to switch between their inactive GDP-bound form and their active GTP-bound form they can amplify or temporise extracellular signals resulting in cytoskeletal rearrangements. Activity of Rho GTPases is under tight control of regulatory proteins, which activate, inactivate or lock the Rho GTPases.

2.2.2. MLC phosphorylation

2.2.2.1 MLC kinases

Rho GTPases regulate cytoskeletal changes involved in cell motility, shape and contraction and are essential in a variety of cardiovascular diseases. The dominant regulatory system of the nonmuscle and smooth muscle F-actin cytoskeleton involves activation of myosin by phosphorylation of the myosin (regulatory) light chains (MLC).[23] Activated myosins bundle F-actin resulting in the formation of F-actin filaments, of which the stress fibers are the most prominent group. Evidence is now accumulating that Rho GTPases are involved in the regulation of MLC phosphorylation and F-actin organization. In order to understand the role of Rho GTPases in MLC phosphorylation it is first necessary to describe the phosphorylation of the MLC.

The myosin II molecule is composed of 2 heavy and 2 distinct light chains, an essential and a regulatory one. Phosphorylation of the regulatory MLC is accomplished by a set of specific kinases, the classical Ca^{2+}-CaM-dependent MLCKs (see Table 1 and ref[71]). Several MLCK isoforms, in the range of 130-150 kD and a splice varient of 210 kD, have been identified. They phosphorylate the MLC both on Ser19 and Thr18. Phosphorylation on these sites increases ATPase activity of the myosin molecule and regulates myosin motor function of the actin/myosin system as its primary function. In the non-phosphorylated folded form, myosin cannot assemble into filaments. Phosphorylation also promotes myosin filament assembly by a conformation change in the myosin molecule. Ser19 is the major site of phosphorylation induced by agonists that stimulate MLC phosphorylation. Under conditions of maximal stimulation Thr18 becomes also phosphorylated. Phosphorylation by eg. PKC on other sites like Ser1, Ser2 or Ser9, does not result in contraction. Substrate specificity of MLCKs is restricted to regulatory light chain of myosin II[71], in contrast to other MLC-phosphorylating kinases including small GTPase-dependent kinases, which often have a broader substrate specificity.

Table 1. Kinases that phosphorylate MLC on Ser19 and/or Thr18

	Ref
130-150 kD MLCK	71;220
210 kD MLCK	221
Embryonic MLCK	72
Endothelial MLCK	73
MAPKAP kinase-2	75
Ca^{2+}/CaM-dependent kinase 2	76
Unidentified, distinct kinase	74
Rho GTPase targets	
Rho kinase (ROCK1/2 or ROKα/β)	77
Pak (Pak1/2/3)	80;81
MRCK(α/β)	82

In search for other MLCKs, Gallagher et al. isolated a developmental regulated MLCK, which was called embryonic MLCK.[72] EmbMLCK seems to be an unique kinase as it is immunologically distinct from the abovementioned 210 kD MLCK. It is also regulated by calcium. In some cells, embryonic MLCK is the predominant form. It is highly expressed in a large variety of ECs (our unpublished observations, 1997). Besides embMLCK, ECs express an endothelial specific MLCK with a molecular weight of 214 kD endothelial MLCK, which seems to be have a role in endothelial permeability.[73]

Recently, evidence was obtained for a calcium-independent MLC kinase distinct from MLCK involved in calcium sensitization of SMC contraction. This kinase phosphorylates MLC on Ser19 or Thr18.[74] Molecular identity of this kinase remains to be elucidated, but is not mitogen-activated protein kinase-activated protein (MAPKAP) kinase-2. MAPKAP kinase-2 exclusively phosphorylates MLC on Ser19 and requires MAP kinase phosphorylation of its activation.[75] Another kinase, that phosphorylates MLC at Ser19, is the Ca^{2+}/CaM-dependent Protein Kinase II.[76] However, it does with a 100 times lower affinity compared to MLCK, so a physiological relevance is not likely.

2.2.2.2 Rho GTPases and MLC phosphorylation

Several mechanisms by which the small G proteins regulate MLC phosphorylation have been elucidated. The first Rho GTPase target shown to involved in MLC phosphorylation was Rho kinase. Rho kinase acts itself as an MLC kinase[77] at least *in vitro*.

Although the apparent K_m value (0.91 µmol/L) of Rho kinase for the MLC is lower than that (52 µmol/L) of MLC kinase, the molecular activity of Rho kinase for MLC is about three times lower than that of MLC kinase. This may be the result of the lower amount of Rho kinase present in cells compared to the amount of MLC kinase as was measured in blood platelets[78] or may be the result of different localization within the cell. Therefore, the Ca^{2+}/CaM-dependent MLC kinase is thought to be the primary regulator of MLC phosphorylation at Ser19. However, contradictory results were obtained regarding the importance of direct phosphorylation of the MLC by Rho kinase (see below).[86]

Rac and Cdc42 activate another family of kinases, the p21-activated kinases (Pak), that is involved in MLC phosphorylation. Three isoforms of Pak have been identified: Pak1 (αPak), Pak2 (γPak) and Pak3 (βPak). In HeLa cells overexpression of Pak reduces MLCK activity, and MLC phosphorylation,[79] and cell spreading was inhibited. *In vitro* it was shown that Pak1 phosphorylates MLCK and inhibits the MLCK activity. This might in part explain the antagonistic effects of Rac and Rho. In contrast to these findings in HeLa cells active Pak2 was shown to increase MLC phosphorylation in ECs.[80] Pak2 phosphorylates the MLC on Ser19, in contrast to MLCK and Rho kinase that phosphorylate MLC both on Ser18 and Thr19. ECs contract upon exposure to Cdc42 or activated Pak2. Pak is required to initiate Ca^{2+}/calmodulin-independent cell retraction. These apparently contradictory findings probably do not reflect Pak isoform differences as other investigators confirmed that Pak increases MLC phosphorylation in ECs using microinjection of active Pak1.[81] A physiological role of Pak remains to be elucidated, but Pak seems to be involved in cell migration.[81]

In a test tube Myotonic Dystrophy Kinase-Related Cdc42-binding kinases (MRCKα/β) can phosphorylate MLC on Ser19. As MRCK is a downstream target of Cdc42, this suggests a role for MRCK in microspike formation.[82] A role of MRCK in intact cells has to be elucidated.

Besides phosphorylating MLC directly, Rho GTPase-dependent kinases regulate MLC phosphate levels by inhibition of the MLC dephosphorylation. As important as the phosphorylation reaction is for the extent of MLC phosphorylation, is the dephosphorylation reaction. It is the ratio between kinase and phosphatase activities that determines the phosphorylation level. MLC dephosphorylation is accomplished by a specific myosin phosphatase, PP1M.[83] Initially it was assumed that the phosphatase activity was a steady one, but now it is known that phosphatase activity is regulated by other factors among which small GTPases. Activity of PP1M is inhibited by phosphorylation of the Myosin Binding Subunit (MBS) of PP1M by Rho kinase.[59;84;85] In many cases inhibition of PP1M accounts for

the major contribution of Rho kinase to elevation of MLC phosphorylation.[78;86] However, in permeabilized vascular smooth muscle both pathways seem to be necessary for an increase in the MLC phosphorylation.[87;88]

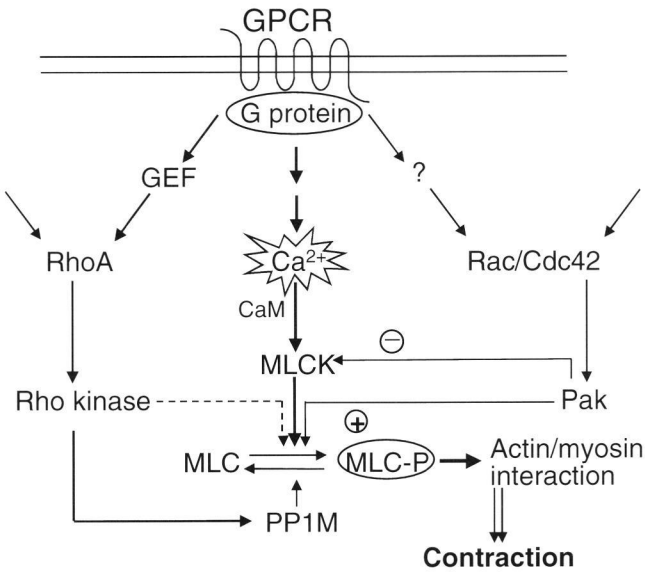

Figure 2. Involvement of Rho-like small GTPases in the regulation of MLC phosphorylation. The common pathway for MLC phosphorylation is a Ca^{2+}/CaM-dependent activation of the MLCK. MLC phosphorylation results in an increased actomyosin interaction and contraction of the cell. Phosphorylated MLC is dephosphorylated by the myosin phosphatase PP1M. At a constant $[Ca^{2+}]_i$ activation of RhoA increases MLC phosphorylation via inhibition of the PP1M by Rho kinase.[86] Rho kinase itself also acts as a MLC kinase *in vitro*. In ECs Pak also phosphorylates the MLC. In some other cell types Pak has been reported to decrease MLC phosphorylation via inhibition of the MLCK. It should be noted that in a test tube the Cdc42-target MRCK also has MLC kinase activity.[82]

In conclusion, cells have an impressive repertoire of activities to increase MLC phosphorylation at their disposal (see Table 1). Furthermore, it has become clear that small GTPases have a profound effect on MLC phosphorylation (see Figure 2). Both Rho, Rac and Cdc42 have a downstream target with MLC kinase activity. Remarkebly less is known about the MLC dephosphorylation. The large repertoire of Rho-like proteins involved in the regulation of MLC phosphorylation is probably related to the involvement of Rho GTPases in many different cellular functions at different cellular sites.

2.2.3. Stress fiber formation

One of the most striking actin structures in the vasculature, which have attracted attention for a long time, are SFs. In endothelial cells *in vivo* SFs occur mainly in large

arteries[89;90], to a lesser extent in the entire microcirculation[91;92], but are largely absent from the venous system.[90;93;94] Many studies have shown that SFs develop during endothelial cell adaptation to unfavorable, or pathological situations including wound healing, atherosclerosis and hypertension.[89;93;95-98] Remarkebly, for each of these conditions evidence has been obtained for the involvement of Rho small GTPases (see below under '*Functional studies*').

As pointed out above, Rho plays an eminent role in the formation of SFs, but how does Rho induce SFs? Many targets of Rho have been identified, among which rhoteckin, rhophilin, PRK2 and citron. The target, which received the major attention, however, is Rho kinase. Activation of Rho kinase results in an increased MLC phosphorylation. This MLC phosphorylation precedes the appearance of SFs and seems to be an early event in SF formation.[23] As outlined above, Rho kinase can phosphorylate the MBS of myosin phosphatase and thereby prevents dephosphorylation of the MLC. Rho kinase can also phosphorylate the MLC directly, but evidence is accumulating, that the former is the most important in most cases. MLC phosphorylation promotes myosin filament assembly and actin-activated myosin ATPase activity, resulting in bundling of actin filaments and the generation of tension.[24]

However, other targets of Rho kinase are also involved in the regulation of SF formation (Figure 3).[99] Rho kinase phosphorylates proteins of the ERM family, promoting their interaction with actin and transmembrane receptors. Phosphorylation of adducin[100] and Na-H exchanger[101] by Rho kinase are also involved in SF formation in a unknown way.

Rho kinase also phosphorylates LIM kinase.[102;103] LIM kinase is a cofilin inactivating kinase.[104] Cofilin exhibits actin-depolymerizing activity. Thus, activation of LIM kinase inhibits depolymerization of F-actin and in this way can promote the formation of SFs. Recently, the picture became even more complicated, as LIM kinase was found also to be activated by Pak kinases.[105;106] This means, that LIM kinase could also be involved in the formation of actin structures induced by Rac and Cdc42.

Furthermore, Rho kinase activity alone is not sufficient for proper SF formation. Expression of a dominant active mutant of Rho kinase induces the formation of stellate SFs, which differ from the parallel SFs found in normal or induced by activation of Rho.[107] Recently, it was shown that an appropiate balance of Rho kinase activity and Dia –another target of Rho- activity can induce SF formation indistinguisable from Rho-induced SF formation. Expression of a dominant active mutant of Dia alone results in weak formation of parallel SFs.[99;107;108] Dia is a profilin-binding protein and probably contributes to SF formation by localizing profilin-bound actin to sites where Rho is active.

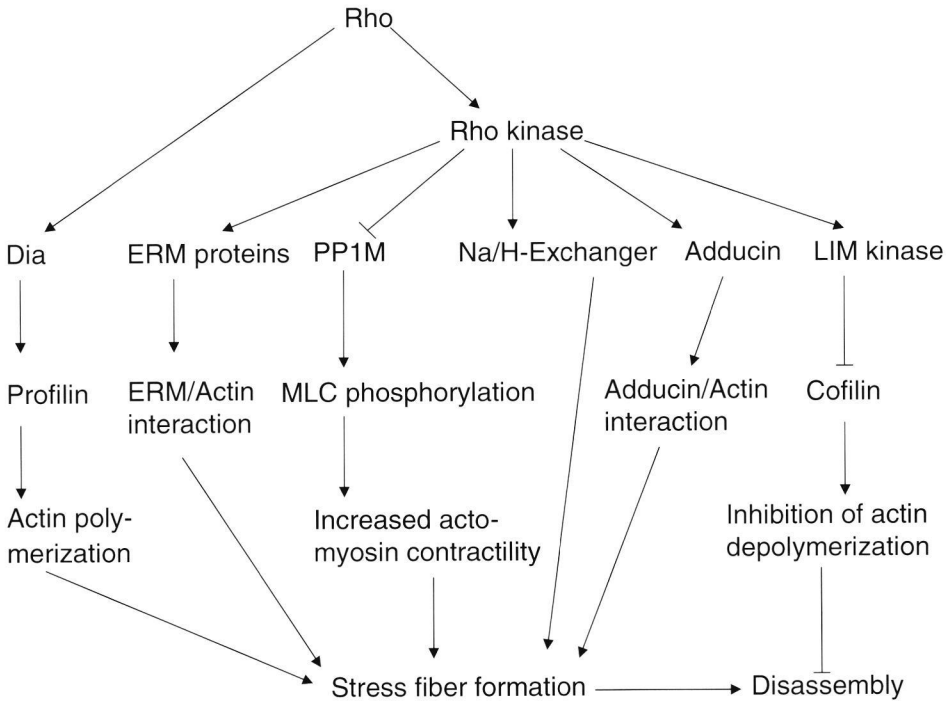

Figure 3. Signal transduction pathways involved in RhoA-induced SF formation. RhoA induces SF formation both by an increased actin polymerization and by an increased interaction between actin and actin-binding proteins, which are required for proper SF formation.[99] Actin polymerization is increased by activation of Dia by RhoA.[107] Activation of LIM kinase enforces this proces by inhibition of the depolymerization of actin.[102] The major target for activated RhoA in SF formation is the Rho kinase. Rho kinase phosphorylates and activates many downstream targets involved in SF formation. Data were combined from references[99;102;107].

An essential feature of Rho GTPases is regulation of MLC phosphorylation. Both Rho, Rac, and Cdc42 have downstream MLC phosphorylating targets, of which the Rho kinase plays an essential role in SF formation. However, Rho kinase activity alone is not sufficient for the proper formation of SFs and other proteins are involved (see Figure 3).

2.3. Functional studies

By regulating the actin cytoskeleton Rho proteins control fundamental processes as contractility, cell shape, cell adhesion and migration. In this section we survey evidence of the involvement of Rho GTPases in the cardiovascular system, focussed on contraction and cell migration. Rho GTPase-mediated contraction is involved in hypertension, arterial

vasospasms, enhanced endothelial permeability, platelet activation and cardiac function. Rho GTPase-mediated motility is involved in (endothelial) wound healing, angiogenesis atherosclerosis, restenosis, and leukocyte and tumor cell transmigration. In phagocytic cells Rac is required for the activation of the a membrane-bound NADPH oxidase in the respiratory burst[109-112], but is beyond the scope of the current review.

2.3.1. Involvement of Rho GTPases in cell contraction and cell shape

2.3.1.1. Smooth muscle cell contraction

Many agents that activate Rho in vascular smooth muscle cells (VSMC) *in vitro* are vasoconstrictors like LPA, thrombin, endothelin and bombesin. In smooth muscle there is strong evidence for the involvement of small GTPases in the contractile mechanism, of which RhoA plays the most prominent role, although other small G proteins, such as Rnd, also have been implicated. In VSMC, it is known that activation of RhoA results in 'calcium sensitization[113]' associated with the tonic component of VSMC contraction, ie, independently of a change in Ca^{2+}, MLC phosphorylation levels and contraction increase upon activation of Rho by vasoactive agents.[86] Rho-mediated Ca^{2+}-sensitization has been demonstrated to be responsible for hypertension in several animal models[114], and to result in coronary artery vasospasm.[115] The role of Rho GTPases in the process of Ca^{2+}-sensitization in VSMC will be described in some detail, as it was first described in VSMC. The same process does not only occur in VSMC contraction, but also in several other vascular processes including platelet activation and endothelial permeability regulation, which have many characteristics in common with VSMC contraction. It also occurs in other types of SMC than VSMC, as *e.g.* Rho is also involved in Ca^{2+}-sensitization in antigen-induced airway hyperresponsive rats.[116]

The dominant regulatory mechanism of nonmuscle and smooth contraction is a Ca^{2+}/CaM-dependent MLC phosphorylation.[117] However, the $[Ca^{2+}]_i$ is not always paralleled by the MLC phosphorylation level. So, other mechanisms in addition to a Ca^{2+}-dependent MLC-phosphorylation must exist. It is firmly established now that Rho plays an important role in Ca^{2+}-sensitization. A decade ago the importance of G proteins in Ca^{2+}-sensitization was suggested from experiments with permeabilized blood vessels. At a constant $[Ca^{2+}]_i$ nonhydrolysable GTP analogs or GTP plus α-adrenergic agonists induced a contraction.[118;119] The involvement of Rho in Ca^{2+}-sensitization was demonstrated using the specific Rho-inhibitor C3-transferase.[87;120] The Ca^{2+}-sensitization is accompanied by a translocation of Rho from the cytoplasm to the cell membrane.[121;122]

The first evidence that Rho kinase mediates the effects of Rho in VSMC contraction came from study of Uehata *et al.*[114], who developed a specific inhibitor of Rho kinase, Y-

27632, and showed that Rho kinase is involved in Ca^{2+}-sensitization induced by a variety of agonists including phenylephrine, thrombin, serotonin, endothelin-1 and the thromboxan agonist U-46619. This finding was confirmed by many other studies.[123-125] Both RhoA and Rho kinase were shown to be present in a variety of VSMC.[126] An interesting observation was that MLC kinase activity is not necessarily required for Rho kinase-induced contraction.[87] Inhibition of Rho kinase did not affect the basal blood pressure, vessel tonus or heart rate.[114;115] *In vitro* studies indicated that Rho kinase can increase MLC phosphorylation both by inhibition of PP1M and by direct MLC phosphorylation.[59;77] In a swine model for coronary artery spasm it was recently demonstrated *in vivo* that Rho kinase was involved in agonist-induced hyperphosphorylation of MLC at Ser19 and Thr18.[115] Using another new inhibitor of Rho kinase, hydroxyfasudil, coronary artery spasm and MLC phosphorylation were reduced.

Besides in contraction, Rho kinase is also involved in VSMC migration and proliferation. These processes are essential for the remodeling of the vessel wall, that also contributes to the development of hypertension, (re)stenosis, and atherosclerosis (see below).[127;128] Interestingly, the Rho-related protein Rnd1 inhibits Ca^{2+}-sensitization of rat smooth muscle, and acted as a natural antagonist of Rho in Ca^{2+}-sensitization.[35] Expression of Rnd1 in VSMC was increased by the sex hormones oestradiol and progesterone. These hormones are known to reduce vascular contractility.[129]

Thus, RhoA/Rho kinase signaling plays an important role in the calcium-independent tonic phase of smooth contraction. It is antagonized by the Rho GTPase Rnd1 at least *in vitro* and is involved in vessel pathologies associated with an hypercontractility.

2.3.1.2. Endothelial permeability

The endothelium, which forms the inner lining of all blood vessels is the main barrier that regulates the extravasation of blood constituents to the surrounding tissues. The most prevalent type of dysfunction of this barrier, which can result in vascular leakage, is the consequence of a contraction process at the margins of endothelial cells (EC) via a Ca^{2+}/CaM-dependent activation of the MLC kinase (reviewed in ref[130-135 136]). This contraction has many characteristics in common with SMC contraction. Rho proteins have been implicated in increased endothelial permeability. Comparably to the involvement of Rho in the the tonic component of VSMC contraction, Rho is involved in cases of prolonged endothelial barrier dysfunction, but not in transient barrier dysfunction induced by histamine and other vasoactive agents in the postcapillary venules.[137-139]

Initial evidence for the involvement of Rho proteins in cell barrier (dys)function came from studies on epithelial cell monolayers.[140-142] The first experiments in EC using the non-selective *Clostridium difficile* toxin B, which inhibits both Rho, Rac and Cdc42, showed that Rho proteins are essential for a proper endothelial barrier function.[143]

Additional experiments showed that the specific Rho-inhibitor C3 exoenzyme inhibited thrombin-induced barrier dysfunction and MLC phosphorylation in HUVEC.[139;144] This indicates that activation of Rho signaling results in a barrier dysfunction. Other Rho-like small G proteins may be involved.[145] However, it is less likely that small GTPases such as Rac and Cdc42 are involved in *in vivo* endothelial barrier dysfunction, as they stimulate cell extension instead of cell contraction. A model was hypothesized in which the transient Ca^{2+}-dependent increase in endothelial permeability can be prolonged or sensitized by activation of RhoA and Rho kinase, similar to 'Ca^{2+}-sensitization' in VSMC. Earlier studies indicated the importance of inactivation of myosin phosphatase by thrombin.[146;147] Essler *et al.* showed an transient inhibition of PP1M by thrombin was Rho-dependent [144]. Rho-mediated endothelial retraction is not restricted to thrombin-induced endothelial permeability, but seems to be involved in many more cases of prolonged endothelial barrier dysfunction. *Pasteurella multocida* toxin and minimally oxidized LDL also induce an endothelial barrier dysfunction via Rho/Rho kinase, even without an increase in $[Ca^{2+}]_i$.[148;149] Leukocytes probably use the same mechanism to transmigrate through endothelial monolayers (see section 'Transmigration of circulating cells'). Interestingly, Siess- *et al.* showed that LPA, a well-known Rho activator, probably is a major active component of ox-LDL with respect to endothelial activation and accumulates in atherosclerotic plaques[150], and LPA was shown to increase endothelial permeability.[151] Endothelial barrier dysfunction is a hallmark of the early atherosclerotic lesion development. This suggests an important contribution of Rho-mediated endothelial barrier dysfunction to the development of atherosclerosis.

In addition to inhibition of the myosin phosphatase by Rho, other mechanisms of Rho action may be involved in endothelial barrier dysfunction. In the case of peroxyvanadate-induced endothelial barrier dysfunction a Rho-mediated activation of the (endothelial) MLC kinase by tyrosine phosphorylation of the MLC kinase has been reported.[152] It remains to be investigated whether vasoactive compounds, like thrombin and LPA, induce such an activation of MLC kinase by tyrosine phosphorylation and whether Rho kinase is involved in MLC kinase activation.

Another function of Rho proteins, which may be involved in the regulation of endothelial barrier function, is regulation of cell-cell interactions. Such a mechanism was demonstrated in epithelial cells.[70;153] However, Braga *et al.*[154] showed that EC are

exceptional in this sense. They demonstrated that in contrast to other cell types Rho activity is not necessary for cadherin-based endothelial cell-cell interaction, and that VE-cadherin localization was insensitive to inhibition of either Rho or Rac. Furthermore, Essler *et al.*[144] showed that inhibition of Rho by C3-transferase did not prevent the thrombin-induced dissociation of catenins from the cytoskeleton. Wojciak-Stothard *et al.*[52] showed that the Cdc42-, Rac- and Rho-dependent TNFα-induced stress fiber formation was also accompanied by an at least partly Cdc42-, Rac- and Rho-independent dispersion of VE-cadherin from intercellular junctions. Thus, at the moment there is no firm support for a role for Rho proteins in the direct regulation of adherens junction organization in EC.

Future studies have to verify whether a similar Rho-induced Ca^{2+}-sensitization mechanism underlies the increased permeability that is enhanced by leukocytes and humoral factors circulating in patients with prolonged edema and in patients undergoing stent implantation.[155]

2.3.1.3. Platelet activation

Similar to VSMC contraction Rho/Rho kinase-signaling has been implicated in MLC phosphorylation in blood platelets.[78;107;156;157] In fact, Rho kinase was first isolated from platelets.[58] Phosphorylation at Ser19 of platelet MLC increases an actomyosin contractile response, that is involved in platelet shape change and secretion. Inhibition of Rho kinase prevented ATP secretion.[78] Several reports indicated that the agonist-induced Ca^{2+}-rise is not required for platelet shape change.[158;159] Rho signaling is also involved in platelet adhesion to fibrinogen.[160] Activation of Rho kinase is accompanied by a translocation of Rho kinase to the actin cytoskeleton.[161] There is evidence that Rho kinase contributes to a great extent to platelet secretion induced by agonists and at low concentrations of thrombin (a strong agonist), but not at high concentrations of thrombin.[156;158] The shape changes induced by weak agonists are fully prevented by inhibition of Rho kinase.

2.3.1.4. Cardiac myocyte hypertrophy

Several reports[162-164] indicated that the Rho/Rho kinase pathway is important for hypertrophic signaling in cardiac myocytes induced by adrenergics, angiotensin II and endothelin-1(reviewed in [165]). A role for the Rho GTPases in myocyte hypertrophy is supported by several recent studies demonstrating the effects of active and inactive forms of RhoA on hypertrophic target gene expression.[162;164;166-168] The effects of RhoA on the morphological and cytoskeletal aspects of the hypertrophy were less clear, with recent reports giving conflicting results.[164;166;169] Similarly, expression of active Rac1 stimulated the hypertrophic program while expression inactive Rac was inhibitory.[163;167]

2.3.2. Involvement of Rho GTPases in cell motility

2.3.2.1. Vascular smooth muscle migration

Many vascular (remodeling) processes depend on motility of vascular cells, which requires a coordinated rearrangement of the actin cytoskeleton and cell-matrix interactions. In cardiovascular diseases, such as hypertension, atherosclerosis and restenosis after angioplasty, vascular remodeling requires changes in the VSMC cytoskeleton.

The migratory response can be induced both by signals from chemoattractants and growth factors as well as by mechanical wounding.[11] In a large variety of cell types an essential role of Rac in cell migration has been established.[11;170] On the one hand Rac is important for the formation of protrusion of lamellipodia at the leading edge of the cells and forward movement and on the other hand Rac seems to be involved in cell retraction at the trailing edge via Pak.[81;171] Cdc42 has been shown to be involved in chemoattractant gradient sensing, ie Cdc42 regulates cell polarity and direction of migration.[172-174] Data concerning the role of Rho in cell migration are still conflicting and may depend on the extend of Rho activation. A low degree of Rho activity is necessary for the generation of adhesive forces and probably for cell retraction.[170;175] A high degree of Rho activity seems to inhibit migration in some cases through formation of strong focal adhesions[11;170], but these studies were performed in nonvascular cells. In VSMC Rho/Rho kinase signaling is involved in cell migration in wound healing assays.[100;127] Recent preliminary data indicate that inhibition of Rho kinase reduces neointima in several animal models and underscore the importance of RhoA/Rho kinase signaling in VSMC migration.[176;177] How the responses of the different GTPases are coordinated remains an interesting area for future research.

2.3.2.2. Endothelial migration and angiogenesis

Comparably to VSMC migration RhoA/Rho kinase signaling has been implicated in endothelial migration.[178-180] Angiogenesis, the formation of new blood vessels from existing ones, is a process, which does not only depend on proliferation, but also on the migration and invasion of ECs. Hla and coworkers recently identified[181] sphingosine-1-phosphate (S1P) as a new angiogenic factor and showed that S1P-induced angiogenesis was completely blocked by inhibition of Rho with C3-transferase. Involvement of Rho-signaling in angiogenesis is not specific for S1P-induced signaling. bFGF-induced *in vitro* angiogenesis is inhibited by the Rho kinase inhibitor Y-27632 (see Figure 4).

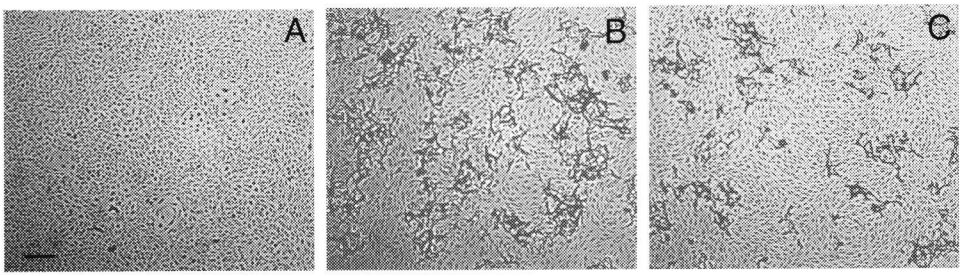

Figure 4. RhoA/Rho kinase signaling is involved in *in vitro* angiogenesis. Human foreskin microvascular ECs were grown on top of a fibrin matrix and stimulated with a cocktail of bFGF and TNFα (B and C) or were not stimulated (A). Stimulation with bFGF/TNFα resulted in the formation of tube-like structures (B). Inhibition of Rho kinase by Y-27632 reduced both the number and the length of the tube-like structures formed after stimulation with bFGF/TNFα. Bar, 300 μm.

This indicates that Rho/Rho kinase-signaling plays an important role in angiogenesis.[182] This brings angiogenesis and endothelial permeability together, as both now appear to depend on similar signal transduction cascades. Vascular leakage is an early manifestion of angiogenesis and results in the extravasation of a fibrinous exudate, providing a provisional matrix for the ingrowth of ECs. It now seems that Rho activation is an ongoing process in angiogenesis and that initial contraction prepares the ECs for migration and ingrowth. Considering the importance of Rac/Pak-signaling in EC migration[81] it is likely that Rac activation also is involved in angiogenesis.

2.3.2.3. Transmigration of circulating cells

For lymphocyte transmigration a multistep model of lymphocyte-endothelial cell recognition and recruitment of lymphocytes from the blood has been proposed in which activation of Rho GTPases plays a central role[183]: (1) Contact through microvillous receptors and rolling of lymphocytes. (2) Activation of lymphocytes through G protein-linked receptors, which trigger (3) integrin adhesion to vascular ligands in seconds through an intracellular pathway involving the small GTP-binding protein Rho, followed by (4) diapedesis. This general model implicates changes in Rho GTPase activity both in the migrating and in the barrier forming (= endothelial) cell. This model also seems to be applicable to leukocyte transmigration and tumor cell invasion.[184-186]

Rho, Rac and Cdc42 regulate the actin cytoskeleton dynamics necessary for chemotaxis of circulating cells.[110;172] Stimulation of leukocytes with fMLP or IL-8 induces a rapid activation of RhoA. In human neutrophils (and eosinophils) fMLP also induces a very rapid and transient activation of Rac.[109] Inhibition of Rho by C3 exoenzyme blocks the adhesion of neutrophils to fibrinogen[187-189], and inhibition of Rho kinase completely inhibits

chemotactic peptide-induced MLC phosphorylation and neutrophil migration.[190] Whereas activation of Rho GTPases is clearly critical for adhesion and migration of circulating cells, the regulation of their activity and the relative individual contribution of each of the distinct GTPases is far from resolved yet. Comparable to transmigration of leukocytes and lymphocytes tumor cell invasion involves RhoA/Rho kinase signaling.[184;191;192]

Evidence is now accumulating that adhesion of circulatory cells to the endothelium directly activates Rho signaling in the endothelium, without the involvement of an intermediate inflammatory mediator. In the multistep model outlined above lymphocyte integrin clustering and adhesion to their counterreceptors on the endothelium takes a central place. Besides in the transmigrating cell, integrin-mediated adhesion can also activate Rho signaling in the endothelial cell, and thus cause endothelial contraction. Activation of Rho in endothelial cells in this way might facilitate the transmigration of these cells across the endothelium[193;194], by creating small pores in the endothelial barrier comparable to those involved in the passage of macromolecules[195], and is accompanied by an increased MLC phosphorylation[196-199] and the formation of SFs[193;195;197;200] (see section on 'endothelial permeability'). In contrast to activation of circulatory cells by chemoattractants, in which both Rho, Rac and Cdc42 are involved, activation of the endothelium by circulatory cells probably involves activation of Rho, but not of Rac and Cdc42.[200]

2.4. Pharmacologic modulation of Rho-like small GTPase signaling

Studies to the function and signaling of Rho-like small GTPases have resulted in the identification of a range of new targets for pharmacological intervention. However, progress in the field of Rho-like GTPase research is hampered by the lack of suitable inhibitors and activators even for *in vitro* work. One of the most important tools currently used in *in vitro* experiments is expression of constitutive active or dominant negative mutants, which are available for Rho[1], Rac[1], Cdc42[32], and their targets Rho kinase[201], Pak[81;202;203], and MRCK.[82] However, their (therapeutic) application is limited as expression of these mutants is prolonged and therefore interferes with all of the functions of the particular protein. It is laborious to use *in vivo* and can not be used under all (experimental) conditions.

Rho proteins are targets for covalent modification by toxins of many pathogenic bacteria (reviewed in[13;204-206]). This suggests an important role for Rho *in vivo*. Among the bacterial toxins several specific activators and inhibitors of Rho function are currently known. Toxin B from *C. difficile* is a general inhibitor of Rho Rac and Cdc42. C3 transferase from *C. botulinum* has a high specificity towards Rho.[204;206] A new development is the *in vivo*

application of C3 transferase in mice via an osmotic minipump.[207] CNF1 from *E. coli* and *Pasteurella multocida* toxin are specific activators of Rho.[148;208]

Several inhibitors with high specificity for Rho kinase compared to MLC kinase and protein kinase C have been developed: Y-27632 and related compounds [114] and fasudil and its hydroxyl-derivative.[115] Both can be used *in vivo* without major effects on basal heart rate and blood pressure and no changes in blood and urine chemistry have been reported sofar.[114;115;185] Y-27632 reduced elevated blood pressure in several animal models for hypertension.[114] Hydroxyfasudil reduced coronary artery vasospasm in a swine model.[115]

Post translational lipid modifications are important both for interactions with GEFs and downstream functions of Rho and are subject of regulation.[16] An exciting new development with therapeutic consequences is the use of statins as inhibitors of Rho function. Statins are inhibitors of the enzyme HMG-CoA-reductase and are used in lipid-lowering therapy. Statins prevent isoprenylation of Rho proteins as a side effect of inhibition of the HMG-CoA-reductase. Isoprenylation (*in casu* geranylgeranylation) is necessary for targetting of RhoA to the plasma membrane.[45;209;210] This may contribute to the nonlipid related beneficial effects of statins, including reduced smooth muscle cell proliferation[211], reduced inflammation and endothelial permeability[212;213], stroke protection[214], and increased fibrinolytic activity.[215] Relatively high concentrations of statins in the micromolar range, however, are necessary to prevent isoprenylation of Rho *in vitro*.[45;209;216;217] The reduction of stroke size by statins in an experimental mouse model via this mechanism underscores the importance of this recognition.[207;214]

2.5. Concluding remarks

Our understanding of the mechanisms by which Rho GTPase activity is regulated and downstream signaling pathways involved has been enormously expanded during the past few years. Involvement of Rho GTPases in many vascular pathologies has been established now. By regulating the actin cytoskeleton Rho proteins control

basal processes including cell migration, contraction, cell shape, and adhesion. Each of these processes is involved in several vascular phenomena: Rho-regulated cell motility is involved in vascular processes including angiogenesis, wound healing, leukocyte transmigration and tumour cell invasion, Rho-regulated contractility is involved in sensitization of Ca^{2+}-induced MLC kinase activity resulting in a prolonged cell contraction in processes including VSMC contraction related to hypertension, endothelial retraction in vascular hyperpermeability and in platelet activation, Rho-regulated cell shape changes are involved in shear stress-induced changes in EC involved in atherosclerosis and restenosis, in

cardiac hypertrophy and platelet activation, and Rho-regulated adhesion is involved in platelet aggregation and leukocyte transmigration (Figure 5).

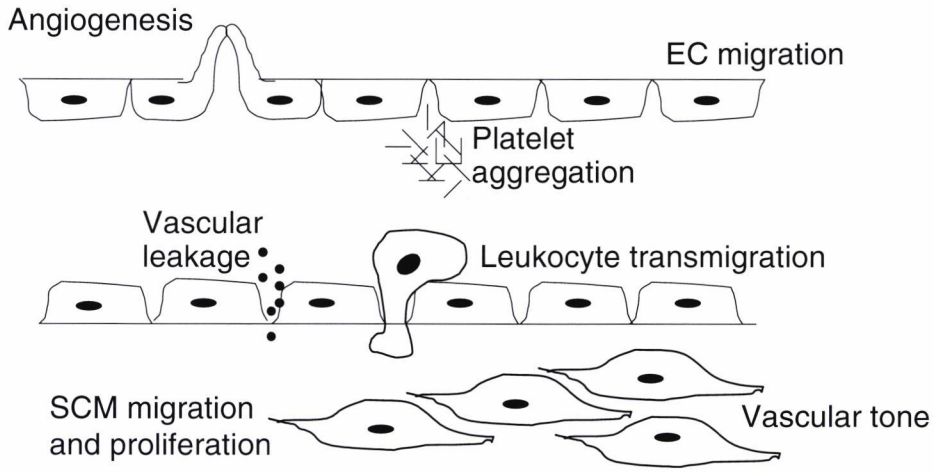

Figure 5. Cartoon indicating the vascular processes in which Rho-like small GTPases are involved.

Our knowledge regarding the precise role of Rho GTPases in these disorders, however, remains fragmentory and we are just in an early stage of learning how these processes are integrated and what the initial triggers are.

One has to be aware that the similar processes can be regulated differently in distinct cell types. This is demonstrated by examples such as: the thrombin-induced Ca^{2+}-mobilization is blocked by inhibition of Rho in fibroblasts, but not in ECs[144;218]; Rho is involved in cadherin function in epithelial cells, but not in ECs[154;219]; phosphorylation of the MLC is induced by Pak in ECs, whereas in HeLa cells MLC kinase activity is reduced by Pak.[79-81]

Studies on Rho-function have resulted in the identification of new targets for pharmacological intervention. The detailed current knowledge of the structure of these proteins will facilitate the development of additional drugs. The discovery that statins inhibit Rho function will obtain clinical application. It remains a future challenge to apply current knowledge of Rho-like GTPase function in treatment of many vascular disorders.

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3

Transient and prolonged increase in endothelial permeability induced by histamine and thrombin.

Role of protein kinases, calcium and RhoA.

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ABSTRACT

In the present study we differentiated between short- and long-term effects of vasoactive compounds on human endothelial permeability in an *in vitro* model. Histamine induced a rapid and transient (<3 min) decrease in barrier function, as evidenced by a decreased transendothelial electrical resistance and an increased passage of ^{22}Na ions. This increase in permeability was completely inhibited by chelation of intracellular calcium ions by BAPTA-AM and by inhibition of calmodulin activity and myosin light-chain (MLC) phosphorylation. The presence of serum factors prolonged the barrier dysfunction induced by histamine.

Thrombin by itself induced a prolonged barrier dysfunction (>30 min) as evidenced by an increased passage of peroxidase and 40 kD dextran. It was dependent only partially on calcium ions and calmodulin. The protein tyrosine kinase (PTK) inhibitors genistein and herbimycin A, but not the inactive analogue daidzein, inhibited to a large extent the increase in permeability induced by thrombin. Genistein and BAPTA-AM inhibited the thrombin-induced permeability in an additive way, causing together an almost complete prevention of the thrombin-induced permeability increase. Inhibition of PTK was accompanied by a decrease in MLC phosphorylation and a reduction in the extent of F-actin fiber and focal attachment formation. Inhibition of RhoA by C3 transferase toxin reduced both the thrombin-induced barrier dysfunction and MLC phosphorylation. Genistein and C3 transferase toxin did not elevate the cellular cAMP levels. No evidence was found for a significant role of protein kinase C in the thrombin-induced increase in permeability or in the accompanying MLC-phosphorylation. These data indicate that in endothelial cell monolayers that respond to histamine in a physiological way, thrombin induces a prolonged increase in permeability by 'calcium sensitization' which involves protein tyrosine phosphorylation and RhoA activation.

INTRODUCTION

The endothelium is the main physical barrier in the extravasation of blood components to the surrounding tissue. Impairment of this barrier leads to an increase in permeability and formation of edema. Inflammatory mediators such as histamine, bradykinin and substance P cause a rapid transient increase in permeability *in vivo*, which results from a rapid formation of endothelial gaps especially in the postcapillary venules.[1; 2] These gaps are thought to be due to endothelial cell (EC) contraction, a process that involves actin non-muscle myosin interaction, and requires Ca^{2+}, calmodulin (CaM) and ATP and the phosphorylation of the myosin light-chain (MLC).[3-5] This process is regulated by Ca^{2+}/CaM-dependent protein kinase I, the classical MLC kinase.[4; 5] Baluk *et al.*[1] recently have shown that the half-life of the gaps induced by substance P in healthy rat tracheal venules is less than two minutes and that the presence of these gaps runs parallel to the increase in endothelial permeability. In frog mesenterium microvessels a similar temporal relationship was found between the transient increase in cytoplasmic Ca^{2+}-ions and the increase in endothelial permeability after stimulation with histamine.[6]

Additional mechanisms must occur to explain the prolonged vascular leakage which contributes to clinical forms of serious edema. These mechanisms include multiple release of vasoactive agents, endothelial binding and activation of leukocytes[7] and, occasionally, the participation of segments of the vascular bed other than postcapillary venules.[8; 9] In these circumstances the endothelial barrier function is reduced by an interplay between actin non-muscle myosin interaction,[4; 5] disintegration of endothelial junctions[10] and the formation of inter- and occasionally intracellular pores.[11-13] However, the molecular mechanisms regulating prolonged leakage are still poorly understood.

Endothelial cells *in vitro* are helpful in providing biochemical information regarding molecular mechanisms contributing to endothelial permeability.[4; 5] Most information has been obtained using thrombin, a stimulus that induces a prolonged increase in permeability in endothelial monolayers,[14] a similar effect to that which it elicits *in vivo*.[15] In contrast to the transient effect of histamine and other vasoactive agents the reduction of endothelial barrier function induced by thrombin extends over one hour and is far beyond the transient rise in cytoplasmic Ca^{2+} concentration that closely accompanies the leakage induced by histamine.[16] Several authors have already pointed to additional factors that influence endothelial permeability other than the Ca^{2+}/CaM-dependent phosphorylation of the MLC. These factors include activation of protein kinase C,[17; 18] and inhibition of MLC phosphatase.[19; 20] In this study we have investigated which pathways are involved in the

different permeability-increasing effects of histamine and thrombin, and provide evidence for the involvement of protein tyrosine phosphorylation and RhoA in addition to the Ca^{2+}/CaM-dependent phosphorylation of MLC in the thrombin-induced permeability of these human endothelial monolayers.

The *in vitro* models have frequently been considered as being inadequate because most studies are limited to prolonged increases in endothelial permeability. Therefore, we also present data on the effect of histamine on human endothelial monolayers. Under the proper conditions, the response to histamine is identical as observed in postcapillary venules *in vivo*; its size and duration can be influenced by exposure of the cells to endotoxin or serum factors.

MATERIALS AND METHODS

Materials

Tissue culture plastics and Transwells (diameter 0.65 cm; pore size 3 μm) were obtained from Costar (Cambridge, MA); cell culture reagents as described previously [21]. Human serum was obtained from the local blood bank and was prepared from fresh blood taken from healthy donors; this was pooled, heat-inactivated (30 min, 56 ^{0}C) and stored at 4 ^{0}C. Heat-inactivated newborn calf serum (NBCS) was obtained from Gibco BRL (Paisley, Scotland). Bovine thrombin 5,000 NIH units was from Leo Pharmaceutical Products (Weesp, The Netherlands). Fluorescein isothiocyanate-labeled dextran with molecular mass 40 kD, histamine, horseradish peroxidase (HRP), lipopolysaccharide from E. coli serotype O128:B12 (LPS), phorbol 12-myristate-13-acetate (PMA), trifluoroperazine (TFP), and anti-vinculin Ig were obtained from Sigma Chemical Company (St. Louis, MO). BAPTA-AM and rhodamine phalloidin were from Molecular Probes (Eugene, OR, USA). ML-7 from Calbiochem Novabiochem Corporation (La Jolla, CA, USA). Calphostin C, genistein, herbimycine A, 1-oleoyl-2-acetylglycerol, thymeleatoxin and tyrphostin A47 were from Alexis Inc. (San Diego, CA). Ranitidine, dimaprit, R-α-methylhistamine and d-neobenodine were a kind gift from Prof H Timmerman (Free University Amsterdam, The Netherlands). Ro31-8220 was a kind gift from Dr PA Brown (Roche Products Ltd, Welwyn Garden, UK). C3 transferase toxin was kindly provided by Dr A Ridley (Ludwig Institute, London, UK). [^{32}P]-orthophosphoric acid and Tran^{35}S label were from ICN Pharmaceuticals, Inc. (Irvine, CA, USA). Anti-platelet myosin Ig (non-muscle) was from Sanbio (Uden, The Netherlands). Rabbit anti-mouse IgG-FITC from Dakopatts (Denmark). ^{22}Na as sodium chloride was from Amersham Lifescience (Buckinghamshire, UK).

Evaluation of the barrier function

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as previously indicated.[21] For the evaluation of the barrier function, confluent monolayers of HUVEC (first and second passage) were released with trypsin-EDTA and seeded in high density on fibronectin-coated polycarbonate filters of the Transwell™ system and cultured. Medium was renewed every other day. Monolayers were used between 4 and 6 days after seeding. Exchange of macromolecules through the

endothelial monolayers was investigated by assay of the transfer of HRP or dextran-FITC and was performed as described previously.[21] All passage experiments were performed in triplicate.
Passage of ^{22}Na, diluted in Medium 199- 1 % HSA to give a specific activity of 1250 cpm/µg, through EC monolayers was examined in a similar way. Passage of ^{22}Na was represented as a difference of histamine-induced and basal passage of ^{22}Na and expressed in cpm/well.

Transendothelial electrical resistance

Transendothelial electrical resistance (TEER) was measured as described previously.[22] In short, an alternating current (50 µA) was passed across the monolayer (2 pulses every minute). The electrical resistance was calculated by Ohm's law and was expressed as a percentage of the basal level. Basal TEER of HUVEC monolayers was 21.3 ± 0.3 $\Omega.cm^2$ (mean ± SEM, 37 determinations in 12 cultures). Basal TEER did not change significantly by pretreatment with the used compounds. 3.10^{-6} mol/L histamine was used, which is intermediate between the half-maximal concentration (~$1.5.10^{-6}$ mol/L) and the maximal effective concentration (10^{-5} mol/L). Histaminergic agonists and antagonists were used at concentrations that were at least ten times higher compared with their pD_2- and pA_2-values.

Phosphorylation of MLC

For analysis of MLC phosphorylation a procedure was adapted from the work of Goeckeler and Wysolmerski[23] and Chrzanowska-Wodnicka and Burridge.[24] Confluent cells of passage 1 or 2, seeded in 12-wells plates, were labeled with 0.5 mL 150 µCi/mL Tran^{35}S-label in low methionine medium (Medium 199 containing 10^{-5} M methionine, 10 % human serum, 10 % NBCS, 150 µg/mL crude endothelial cell growth factor, 5 U/mL heparin, 100 U/mL penicillin and 0.1 mg/mL streptomycin) for 48 hours. Cells were washed with phosphate-free buffer (119 mmol/L NaCl, 5 mmol/L KCl, 5.6 mmol/L glucose, 0.4 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 25 mmol/L Pipes (pH 7.2)), and labeled with 0.5 mL 150 µCi/mL [^{32}P]orthophosphoric acid in phosphate-free buffer for 2 hours and then stimulated with histamine or thrombin. Buffer was removed and cells were lysed by scraping in 300 µL of ice-cold lysis-buffer (25 mmol/L Tris-HCl, 250 mmol/L NaCl, 75 mmol/L NaF, 5 mmol/L EGTA, 5 mmol/L EDTA, 1 % NonidetP-40, 0.5% sodiumdeoxycholate, 0.2 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L dithiothreitol, 10 µg/mL aprotinin, 100 mmol/L $Na_4P_2O_7$). The lysates were centrifuged for 20 min in an Eppendorf centrifuge. The supernatants were incubated with 6 µL of polyclonal rabbit anti-platelet myosin IgG for 1 hour. Subsequently, 20 µL (1:1) of prewashed protein A-Sepharose 4B was added for an additional hour. Immune complexes bound to protein A Sepharose 4B were collected by centrifugation. Pellets were washed 3 times with PBS and resolved in 50 µL SDS sample buffer. The samples were electrophoresed on 16 % SDS-polyacrylamide gels. The gels were dried and exposed to a phosphoimaging screen. Quantitation of ^{32}P incorporation into MLC was performed using a Fuji BAS 1000 phosphoimager as follows. Double-labeled samples were exposed to phosphoimaging screens directly and the total amount of radioactivity (^{35}S + ^{32}P) was quantitated for MLCs. A second exposure was obtained in which a filter of 4 layers of aluminum foil was present between the gel and the phosphoimaging screen to block ^{35}S radiation. The amount of ^{32}P and ^{35}S incorporation in the MLC band of each sample was calculated, and MLC phosphorylation was expressed as a percentage of control.

Extraction and assay of cyclic AMP

Intracellular cAMP levels were determined by radio-immunoassay (Amersham, Amersham, UK) as described previously.[21]

Immunocytochemistry

The presence of vinculin and F-actin were visualized by indirect immunofluorescence with mouse anti-vinculin antibody (1:300) and by direct staining with rhodamine-phalloidin (1:100).

Statistical analysis

Data are reported as mean ± SEM (standard error of the mean). Comparisons between > 2 groups were made using the Kruskal-Wallis test, and individual groups comparisons were done using a Mann-Whitney test for post hoc comparisons. Comparisons of time curves of two groups were made using repeated measures ANOVA and individual groups comparisons were done using a Student t test for post hoc comparisons of the means. Differences were considered significant at the $P < 0.05$ level.

RESULTS

Histamine induces a short-term decrease in endothelial barrier function.

In HUVEC, the addition of histamine (3.10^{-6} mol/L) induced a rapid decrease in the real-time transendothelial electrical resistance (TEER), that was maximal after 1 min (72 % ± 7 as compared to basal level, 5 different cultures in triplicate) and lasted for < 5 min (see Figure 1A). An increase in the passage of ^{22}Na paralleled the decrease in TEER (see Figure 1C). The effect of histamine on TEER was mimicked by the histamine H_1-agonist thiazolylethylamine, but not by the histamine H_2-agonist dimaprit (see Figure 1B). Preincubation for 10 min with the histamine H_1-antagonist d-neobenodine blocked the decrease in TEER induced by histamine, while the histamine H_2-antagonist ranitidine had no effect (see Figure 1A).

The histamine-induced increase in EC permeability was inhibited completely by preincubation with the intracellular calcium chelator BAPTA-AM or the calmodulin inhibitor TFP (see Figure 1D) and to a large part by the MLC kinase inhibitor ML-7 (see Figure 1E). Addition of histamine to HUVECs increased MLC phosphorylation transiently (135 ± 13 % after 2 min, see Figure 2, closed symbols). This effect extended slightly beyond the decrease in TEER. However, part of the MLC phosphorylation was inhibitable by the protein tyrosine kinase (PTK) inhibitor genistein (30 µg/mL for 1 hour). The genistein-insensitive MLC phosphorylation exactly paralleled the histamine-induced increase in permeability (see Figure 2, open symbols), as the histamine induced decrease in TEER was completely insensitive to inhibition of PTKs with genistein (see Figure 1F). Preincubation of the cells with C3 transferase did not affect the histamine-induced increase in permeability, (see below).

Together, these data are consistent with a role of Ca^{2+}/CaM-dependent and genistein-insensitive phosphorylation of MLC in the transient increase in permeability induced by histamine.

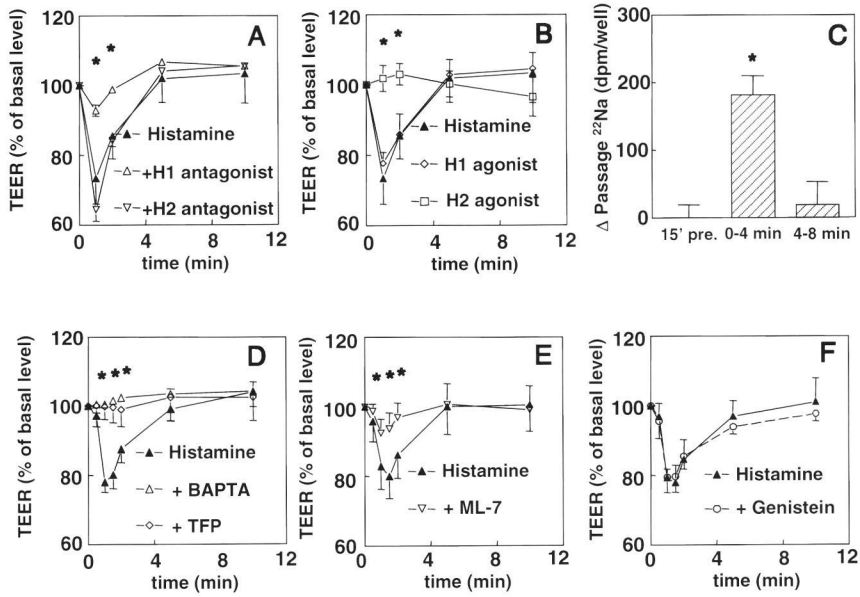

Figure 1. Histamine transiently decreases the barrier function of human umbilical vein endothelial monolayers by a Ca^{2+}/CaM- and MLCK-dependent mechanism.

A and B, Effect of histaminergic compounds on the TEER. A. Cells were pretreated for 10 minutes with 3.10^{-8} mol/L d-neobenodine (H_1-antagonist), 10^{-6} mol/L ranitidine (H_2-antagonist) or were sham-treated and subsequently stimulated with 3.10^{-6} mol/L histamine. Values are the mean ± SEM of two different cultures in triplicate. * $P<0.05$, d-neobenodine-pretreated cells versus control cells. Basal TEER of HUVEC monolayers was 21.3 ± 0.3 $\Omega.cm^2$ (mean ± SEM, 37 determinations in 12 cultures). B, Cells were equilibrated for 10 minutes and stimulated with 3.10^{-6} mol/L histamine, 10^{-5} mol/L thiazolylethylamine (H_1-agonist) or 1.10^{-5} M dimaprit (H_2-agonist).Values are the mean ± SEM of 2 different cultures in triplicate. * $P<0.05$, dimaprit- versus histamine-stimulated cells.

C, Effect of histamine on the passage of ^{22}Na. Monolayers were equilibrated for 15 minutes and at 0 minutes were stimulated with 10^{-5} mol/L histamine or sham-treated. Passage of ^{22}Na was expressed as a difference between histamine-stimulated and basal passage of ^{22}Na. Values are the mean ± SEM of two different cultures in 6-fold. * $P<0.05$, histamine-induced versus basal ^{22}Na passage.

D, E, and F, Effect of various inhibitors on the histamine-induced decrease in TEER. D, Cells were pretreated for 1 hour with 3 µmol/L BAPTA-AM (open triangles), 10 µmol/L TFP (diamonds) or sham-treated (closed triangles). After 1 hour cells were exposed to 3.10^{-6} mol/L histamine. Values are the mean ± SEM of 2 different cultures at least in triplicate. * $P<0.05$, BAPTA-AM- or TFP-pretreated versus control cells. E, Cells were pretreated for 2 hours with 10 µmol/L ML-7 (open triangles) or sham-treated (filled triangles). After 2 hours cells were stimulated with 3.10^{-6} mol/L histamine. Values are the mean ± SEM of 2 different cultures in triplicate. * $P<0.05$, ML-7-pretreated versus control cells.

F, Cells were pretreated with 30 µg/mL genistein for 1 hour (open circles) or sham-treated (closed triangles). After 1 hour cells were stimulated with 3.10^{-6} mol/L histamine. Values are the mean ± SEM of 2 different cultures at least in triplicate.

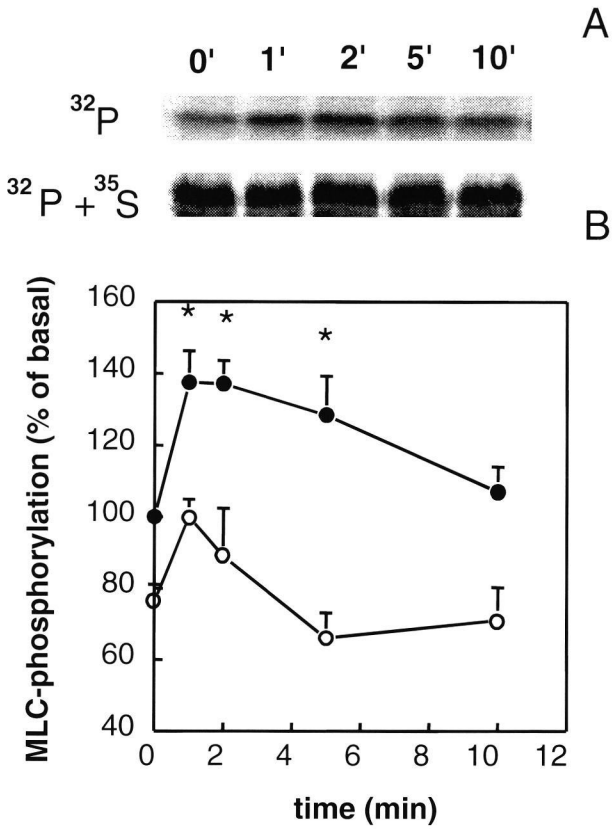

Figure 2. MLC phosphorylation is stimulated transiently by histamine.

A, Autoradiograph of MLCs immunoprecipitated from cells under basal conditions (0 minutes) and 1, 2, 5 and 10 minutes after stimulation with 10^{-5} mol/L histamine. Cells were labeled with ^{32}P and ^{35}S as described in Materials and Methods. Top, An exposure in which a filter was present to block ^{35}S signal; Bottom, An exposure without filter.

B, Quantitation of MLC phosphorylation. Cells were preincubated for 1 hour in the absence (closed circles) or the presence of 30 µg/mL genistein (open circles), stimulated with 10^{-5} mol/L histamine, and MLC phosphorylation was measured after the time points indicated. The level of ^{32}P incorporation into MLCs was calculated relative to the amount of ^{35}S incorporation into the MLCs of the same sample, as described in Materials and Methods. Histamine significantly induced MLC phosphorylation at time points 1, 2, and 5 minutes (* $P<0.05$). Genistein significant lowered MLC phosphorylation ($p=0.000$). Values were the mean ± SD of 2 different cultures at least in duplicate.

The response of endothelial cells to histamine can be modified.

Pretreatment of HUVECs with 10 µg/mL LPS for 24 hours enlarged the decrease in TEER induced by histamine (see Figure 3A) from 79 % ± 9 to 58 % ± 5 (mean ± SEM of 3 different cultures at least in triplicate, $P<0.001$). Addition of human serum to our system considerably prolonged the response induced by histamine (see Figure 3B).

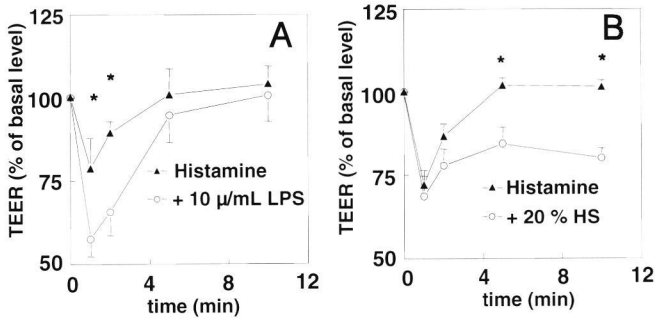

Figure 3. Effect of LPS and human serum on the histamine-induced decrease in TEER.
A, Endothelial monolayers were grown to confluence and cultured for 24 hours in absence (triangles) or presence of 10 µg/mL LPS (circles). Cells were stimulated with 3.10^{-6} mol/L histamine.
B, Cells were stimulated with 3.10^{-6} mol/L histamine in the absence (triangles) or presence of 20% human serum and TEER was recorded. Values are the mean ± SD of at least 2 experiments in triplicate. * $P<0.05$, pretreated versus control cells.

Thrombin induces a prolonged decrease in endothelial barrier function that involves PTK activity.

A prolonged response is also induced by thrombin in the absence of serum. The thrombin-induced reduction in TEER lasted for at least 30 minutes. The decrease in TEER was paralleled by a rapid increase in the passage of macromolecules, which was usually sustained for at least 1 hour, as is shown for HRP in Figure 4.

In agreement with previous reports [21; 25] the prolonged increase in permeability for macromolecules (HRP) induced by thrombin was partially inhibited (maximally 60%) by BAPTA-AM (see below) or by the calmodulin inhibitor TFP (not shown). Thus, in contrast to the transient increase in permeability induced by histamine, the prolonged increase induced by thrombin required additional activation or sensitization steps other than Ca^{2+}/CaM-dependent MLC phosphorylation. Therefore, we evaluated whether inhibition of PKC or PTK affected the thrombin-induced permeability.

At 1 and 10 nmol/L the PKC activator PMA (15 minutes' preincubation) reduced endothelial permeability by 25 to 50%, whereas at higher concentrations (100 nmol/L), which give an extreme activation of PKC as compared to thrombin and histamine, it increased the permeability. In the presence of thrombin, 10 nmol/L PMA reduced the permeability in HUVEC significantly (Table 1). Similarly, the PKC activating diacylglycerol analogue 1-oleoyl-2-acetylglycerol (50 µmol/L) and thymeleatoxin (10 nmol/L), which activates predominantly calcium-dependent PKCs, reduced thrombin-enhanced permeability of dextran by 42 ± 13 % (n=7) and 44 ± 6 % (n=6), respectively.

Table 1: Passage of dextran (40 kD) through HUVEC monolayers and MLC phosphorylation in the presence or absence of PMA, Ro31-8220 and/or thrombin.

	Passage of dextran ($\mu g.h^{-1}.cm^{-2}$)		MLC phosphorylation (% of basal level)
Addition	Control	+Thrombin	+Thrombin
None	1.3 ± 0.2	14.0 ± 3.4	165 ± 15
Ro31-8220	2.3 ± 0.5	14.4 ± 4.9	148 ± 18
PMA	1.0 ± 0.2*	6.2 ± 1.5*	143 ± 12
PMA+Ro31-8220	2.4 ± 0.5*#	15.4 ± 2.4#	156 ± 19

HUVEC were preincubated under basal conditions with Ro31-8220 (1 µmol/L) 15 min prior to PMA (10 nmol/L); PMA (10 nmol/L) or Ro31-8220 were added 15 min before thrombin (1 U/mL). The passage of FITC-labeled dextran (40 kD) was determined after one hour (thrombin) or two hours (basal) incubation. Values are the mean ± SEM of six different cultures in triplicate; *P<0.05 vs. corresponding control value; #P<0.05 vs. corresponding PMA value. MLC phosphorylation was determined as described in Material & Methods and is expressed as the mean percentage ± SEM of basal MLC phosphorylation of two different cultures in duplicate.

Subsequently, the PKC inhibitors Ro31-8220 and calphostin C were used. Although Ro31-8220 completely counteracted the effect of PMA, it did not alter the thrombin-induced increase in permeability (see Table 1). Calphostin C (100 nmol/L) slightly increased the basal permeability (from 0.82 ± 0.17 to 1.21 ± 0.36 $\mu g.h^{-1}.cm^{-2}$), but the thrombin-stimulated permeability was not affected by 15 minutes' preincubation with calphostin C , being 7.8 ± 1.7 $\mu g.h^{-1}.cm^{-2}$ and 7.2 ± 1.4 $\mu g.h^{-1}.cm^{-2}$, respectively (4 cultures). The phosphorylation of MLC was not affected by PMA or Ro31-8220 under thrombin-stimulated conditions (see Table 1). These data do not support a significant role for PKC activation in thrombin-induced increase in permeability of HUVEC monolayers.

Subsequently, we studied whether inhibition of PTK prevented the thrombin-enhanced permeability. As shown in Figure 4, preincubation of HUVEC monolayers with genistein, but not its inactive analogue daidzein (both preincubated at 30 µg/mL for 1 hour) attenuated the thrombin-induced HRP passage, which became significant after 5 minutes. The reduction of thrombin-induced permeability by genistein was dose-dependent (3 to 100 µg/mL tested, data not shown). Preincubation with another PTK inhibitor herbimycine A (0.3 µg/mL for 2 hours) resulted in a similar inhibition, whereas tyrphostin A47 (up to 10 µg/mL), which has a different substrate spectrum, had no effect on the thrombin-induced HRP passage.

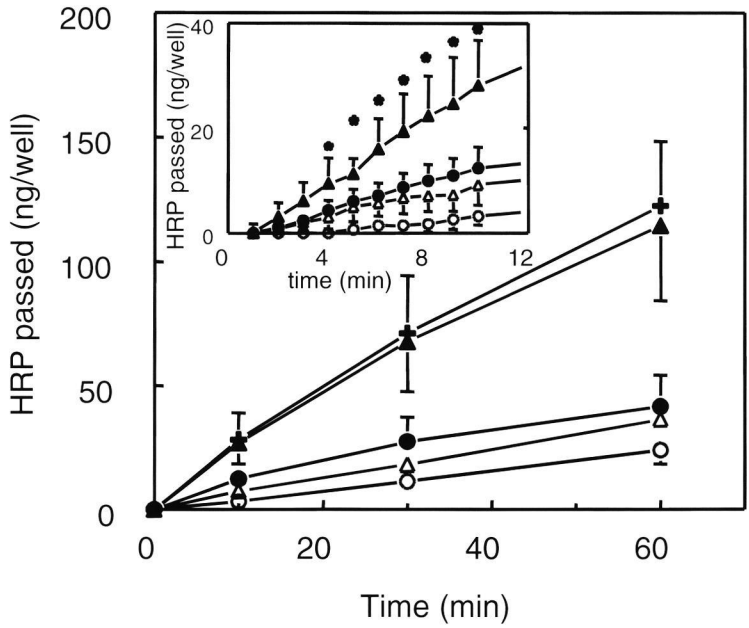

Figure 4. Effect of genistein on the passage of HRP through human umbilical vein endothelial monolayers under basal conditions (open symbols) and after exposure to 1 U/mL thrombin (closed symbols). Cells were preincubated for 1 hour in Medium 199-1% HSA with 30 µg/mL genistein (circles) or with 30 µg/mL daidzein (crosses) or without addition (triangles). The inset shows the early timepoints of the same set of experiments in more detail. Values are the mean ± SD of 2 different cultures in triplicate. The interaction between time and genistein treatment was significant (p=0.000) for thrombin-stimulated cells. Treatment with genistein resulted in lower HRP passage compared to no treatment. Differences were significant from 4 minutes onward. * P<0.05, genistein-pretreated versus non-pretreated cells, that were stimulated with thrombin.

Genistein reduced the basal MLC phosphorylation (see Figure 5), parallel to its reduction of basal permeability. Thrombin induced a prolonged increase in the phosphorylation of MLC (see Figure 5 inset, closed symbols), which was maximal 1 minute after exposure to thrombin and remained elevated for at least 30 minutes. Although the degree of MLC phosphorylation in thrombin-induced HUVEC was lower in genistein-treated cells, this decrease reflected the lower basal MLC phosphorylation state rather than the increase induced by thrombin itself (Figure 5 inset, open symbols). Simultaneously, the thrombin-induced decrease in TEER attenuated by 36 ± 17 % (assayed after t=10 minutes, P<0.01, 2 experiments in 5-fold) after preincubation of 30 µg/mL genistein, whereas genistein did not affect the basal TEER.

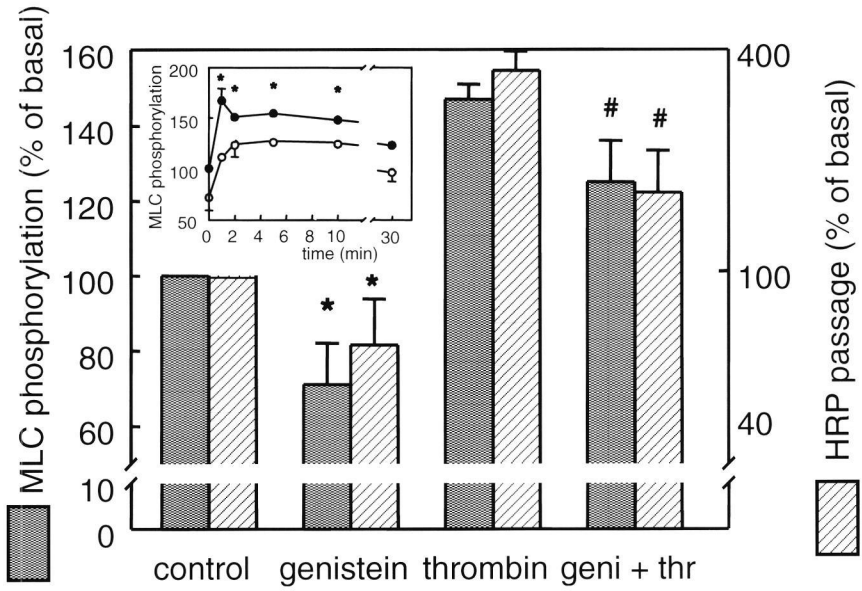

Figure 5. Parallel inhibition of MLC phosphorylation and HRP passage by genistein. Cells were preincubated for 1 hour in the absence or presence of 30 µg/mL genistein, stimulated with 1 U/mL thrombin and MLC phosphorylation was determined after 10 minutes (cross-hatched bars). HRP passage data are derived from Figure 4 and represent the amount of HRP passed after 10 minutes and are expressed as a percentage of basal level (hatched bars). * $P<0.05$, genistein-pretreated versus control cells. # $P<0.05$ thrombin-stimulated cells in the presence versus absence of genistein. *Inset* Time curve of inhibition by genistein of thrombin-induced MLC phosphorylation. Cells were preincubated for 1 hour in the absence (closed triangles) or presence of 30 µg/mL genistein (open triangles), and stimulated with 1 U/mL thrombin. MLC phosphorylation was determined after the time points indicated. MLC phosphorylation was measured as described in Materials and Methods and expressed as a percentage of basal MLC phosphorylation. Thrombin significantly induced MLC phosphorylation at 1, 2, 5 and 10 minutes (* $P<0.05$). Genistein significant lowered MLC-phosphorylation ($p=0.001$). Values are the mean ± SD of 2 different cultures at least in duplicate.

When HUVEC were preincubated with genistein, the cellular cAMP concentration remained unaltered both under basal conditions (2.3 ± 0.2 and 2.3 ± 0.1 pmol/3.5x10^5 cells in control and genistein-preincubated cells) and after 10 minutes stimulation of the cells with 1 U/mL thrombin (3.1 ± 0.5 and 2.9 ± 0.3 pmol/3.5x10^5 cells, respectively; 3 experiments). This excludes the possibility that genistein could act on endothelial permeability and MLC phosphorylation via elevation of the cAMP concentration.

The inhibition of thrombin-induced permeability by genistein was additive to that by BAPTA-AM (see Figure 6). Together these compounds caused a nearly complete prevention of the thrombin-induced permeability. They also affected the basal permeability, probably because a low degree of endothelial activation occurs even under basal conditions. These data show that Ca^{2+}-dependent and PTK-sensitive pathways cooperate in the regulation of endothelial permeability.

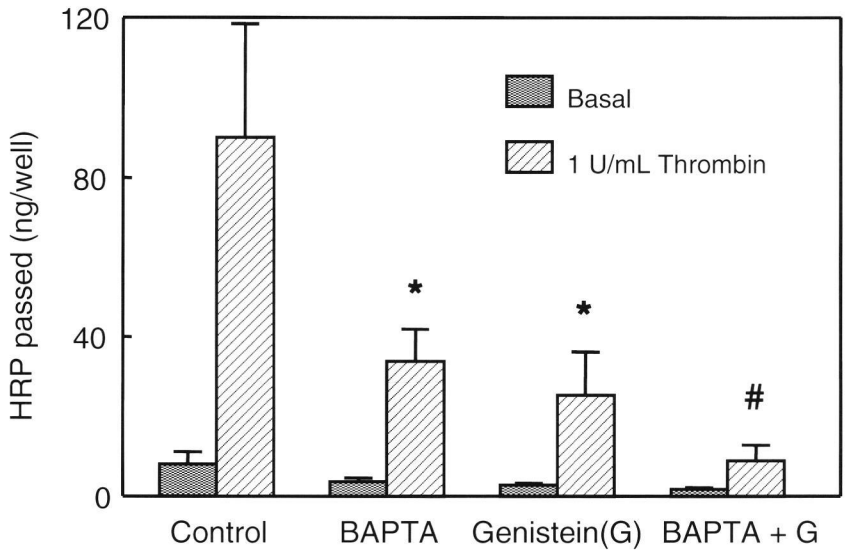

Figure 6. Effect of BAPTA-AM and genistein on basal (cross-hatched bars) and thrombin-enhanced permeability (hatched bars). Endothelial monolayers were pretreated for 1 hour with 3 µmol/L BAPTA-AM, 30 µg/mL genistein, or a combination of both. The HRP passage was determined 1 hour after sham treatment or exposure to 1 U/mL thrombin. Values are the mean ± SEM of three different cultures in triplicate. There was a significant difference between HRP passage of the various thrombin-stimulated groups ($P=0.0008$). * $P<0.05$, cells pretreated with BAPTA-AM or genistein versus non-pretreated cells. # $P<0.05$, cells pretreated with a combination of BAPTA-AM and genistein versus pretreatment with each compound alone.

PTKs are involved in thrombin-induced cytoskeletal rearrangements

Under basal conditions F-actin fibers in HUVEC were arranged into a fine cortical network with a dense peripheral band evident at the cell boundaries (see Figure 7A); occasionally stress fibers were observed. When the monolayers were preincubated with genistein (30 µg/mL, see figure 7B) the cells became wrinkled, the cortical actin band was less obvious and an increase in diffuse cytoplasmic actin staining was observed. After stimulation of the

cells with 1 U/mL thrombin many stress fibers were formed (see Figure 7C), simultaneously small gaps became visible at the cell-cell contact areas and an increase in vinculin staining was observed, indicating that focal adhesion sites were formed (see Figure 7E and 7G). The thrombin-induced cytoskeletal rearrangements were inhibited largely by preincubation with genistein (see Figure 7D and 7H). This was reflected by a marked, but not complete reduction of stress fibers, a reduction of the occurrence of small gaps, and a reduction in the extent of focal adhesion formation.

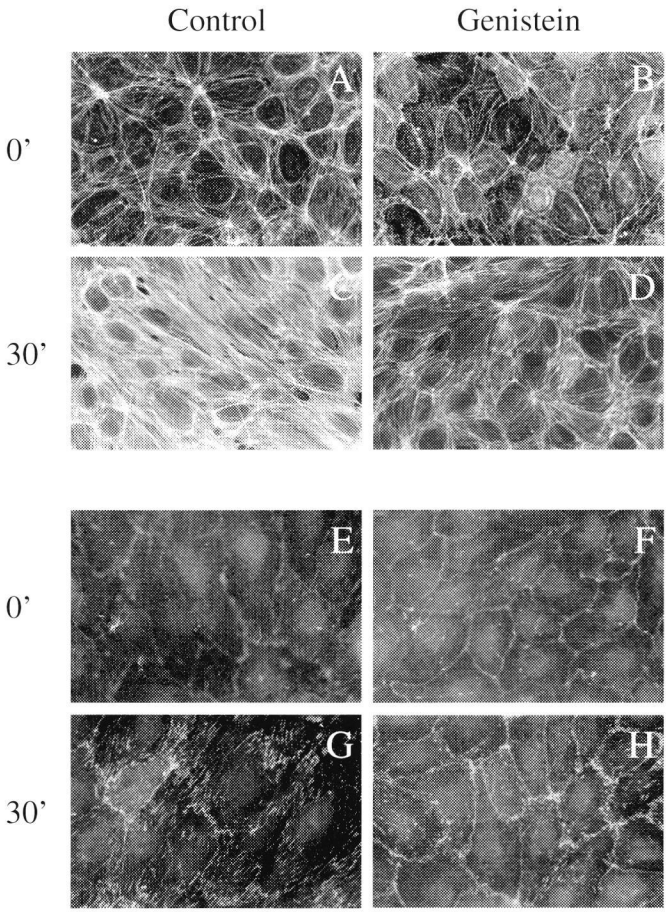

Figure 7. Immunocytochemical staining of actin (A through D) and vinculin (E through H) in HUVEC. ECs were preincubated for 1 hour with 30 µg/mL genistein and stimulated with 1 U/mL thrombin. The cells were stained as described in Materials and Methods.
A and E, Basal condition. B, D, F and H, Cells were preincubated with genistein. C, D, G and H, Cells were stimulated for 30 minutes with thrombin.

RhoA involvement in thrombin-enhanced permeability

Recent data pointed to a role of RhoA in the formation of stress fibers[26] and cell contraction[24; 27; 28] in non-vascular cells. Because thrombin-induced tyrosine phosphorylation may be involved in the activation of RhoA and subsequently in the regulation of stress fiber formation via RhoA, we used an inhibitor of RhoA, the toxin C3 transferase of *C. botulinum* to evaluate whether RhoA contributed to the thrombin-induced increase in permeability. Titration of the C3 transferase toxin revealed that 24 hours' incubation with 3 to 5 µg/mL toxin caused sufficient uptake without changing the permeability of the monolayers by itself. Preincubation with 5 µg/mL C3 transferase toxin markedly attenuated the thrombin-enhanced permeability as well as basal permeability (see Figure 8A). This attenuation was accompanied by a reduced MLC phosphorylation (see Figure 8B). The reduction in endothelial permeability by C3 transferase was abolished by heat treatment of the C3 transferase preparation (5 minutes at 95 ^{0}C). Preincubation of the cells with 5 µg/mL C3 transferase did not change the cAMP level of thrombin-stimulated cells (2.5 ± 0.6 versus 2.6 ± 0.3 pmol/3.5x10^{5} cells, 2 cultures in duplicate).

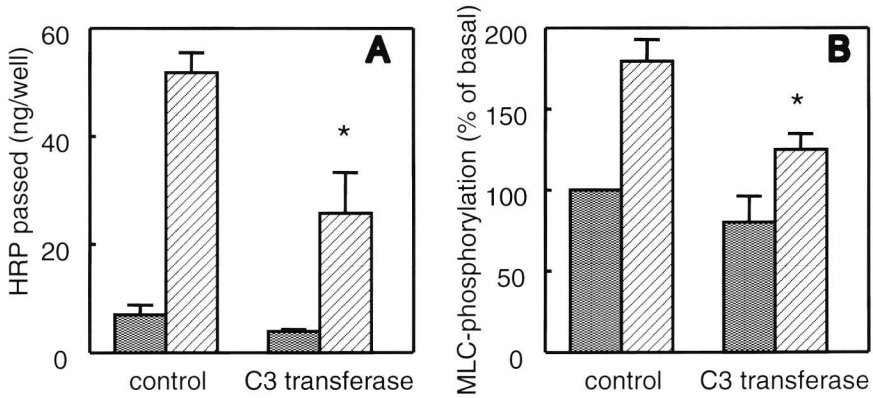

Figure 8. Effect of C3 transferase on basal (cross-hatched bars) and thrombin-enhanced (hatched bars) permeability (A) and MLC-phosphorylation (B). Endothelial monolayers were pretreated for 24 hours with 5 µg/mL C3 transferase. The HRP passage was determined 1 hour after sham treatment or exposure to 1 U/mL thrombin. MLC phosphorylation was measured as described in Materials and Methods 10 minutes after addition of thrombin. Values are the mean ± SD of 2 different cultures at least in duplicate. * P<0.05, thrombin-stimulated cells that were pretreated with C3-transferase versus non-pretreated cells.

DISCUSSION

In the present study, we have provided evidence for the involvement of PTK activity and RhoA in the prolonged thrombin-induced increase in permeability. The thrombin-enhanced permeability was accompanied by an increase in MLC phosphorylation and the generation of F-actin fibers, both of which were reduced by the inhibition of PTK or RhoA. The transient increase in permeability induced by histamine was not affected by PTK and RhoA inhibition.

In *in vivo* experiments stimulation of healthy vessels with vasoactive agents, such as histamine, bradykinin and substance P, induces a transient increase in permeability of postcapillary venules.[1; 2] This involves a Ca^{2+}/CaM-dependent activation of the EC[6] and a transient formation of minute gaps at the cell-cell contacts leaking monastral blue during a period of only a few minutes.[1] In our *in vitro* model the time course of the permeability increase induced by histamine is identical to the increase observed *in vivo* in leakage and involves a similar activation mechanism.[6] However, under many pathological conditions massive leakage can occur over a long period of time. We have demonstrated here that the increase in endothelial permeability induced by histamine was significantly prolonged in time by the addition of serum factors. These data support the idea that additional mechanisms contribute to the prolonged vascular leakage. At this moment, the additional factor(s) in serum responsible for the prolongation of the histamine effect are not yet known. Recently, Bloemers *et al.*[29] reported a sensitization of H_1-receptor in embryonic cells by the presence of serum and leukotrienes. Alternatively, sensitization of the Ca^{2+}/CaM-dependent activation of MLC phosphorylation, known from studies in smooth muscle cells,[28] may also contributes to the prolonged response.

The thrombin-induced increase in endothelial permeability provides a condition in which the Ca^{2+}/CaM-dependent MLC phosphorylation is sensitized. Chelation of cytoplasmic Ca^{2+}-ions by BAPTA-AM only partially reduces the thrombin-induced increase in endothelial permeability.[21; 25] Our data show that inhibition of PTK reduces thrombin-induced permeability by an additional mechanism and almost completely prevents this increase when added simultaneously with BAPTA-AM. Inhibition of PTK may act on endothelial permeability by various mechanisms. It has been shown that PTK inhibitors attenuate the agonist-induced cellular Ca^{2+} influx.[30; 31] However, our finding that coincubation of BAPTA-AM with genistein has an additive effect suggests that activation of PTKs by thrombin involves an additional pathway. Other reports have pointed to the disruption of adherens junctions and reorganization of focal adhesion plaques by thrombin. Rabiet *et al.*[32] found that thrombin

disrupted the V,E-cadherin-catenin complex in adherens junctions, which could be prevented by the PTK inhibitor herbimycin A. Schaphorst *et al.*[33] reported the tyrosine phosphorylation of focal adhesion kinase pp125^{FAK}, which accompanied the reorganization of focal adhesion sites induced by thrombin. These changes in cell-cell and cell-matrix interaction may contribute to the enhanced permeability induced by thrombin. Here, we show that inhibition of PTKs affects the formation of F-actin fibres and decreases the MLC phosphorylation. By these actions it may influence the interaction of the F-actin cytoskeleton with cell-cell and cell-matrix attachment sites and reduces actin-non muscle myosin interaction causing local contractile forces probably in the margins of the ECs.

Another candidate molecule to be regulated by PTKs and involved in permeability is RhoA. Hippenstiel *et al.*[34] recently have shown that RhoA plays a role in basal endothelial permeability. A well-established effect of RhoA is stress fiber formation, a process that is known to be activated by PTKs in ECs.[35] Furthermore, it is known that activation of RhoA induces MLC-phosphorylation, probably via Rho kinase, which may act via inactivating the regulatory subunit of a myosin phosphatase.[36] Here we show that inhibition of RhoA by C3 transferase attenuates both the basal and thrombin-induced EC barrier dysfunction and the accompanied increase in MLC-phosphorylation. This fits with the finding that the thrombin-mediated stress fiber formation is dependent on the activation of PTKs. The function of these stress fibers is not completely understood at present. In ECs they seem to have a protective function against fluid shear stress by developing cell tension.[37] Goeckeler and Wysolmerski[23] and Moy *et al.*[16] have demonstrated that tensile forces are developed in thrombin-stimulated ECs, whereas these forces are insignificant in HUVEC exposed solely to histamine. In this context it is likely that the F-actin fiber structures contribute to a state of prolonged EC contraction after thrombin challenge. Furthermore, it is known that in smooth muscle cells activation of RhoA results in 'calcium sensitization', i.e. independently of a change in Ca^{2+}, MLC phosphate levels increase by inhibition of the smooth muscle myosin phosphatase (SMPP-1) via activation of a RhoA-dependent kinase, resulting in force generation.[28] In addition to an effect of Rho-kinase on the activity of myosin phosphatase 1, Rho kinase may also act itself as a MLC kinase.[36] In smooth muscle cells, the calcium sensitization is accompanied by a translocation of RhoA from the cytosol to the cell membrane.[28] Preliminary data indicate that activation of ECs by thrombin results in a similar translocation of RhoA. Verin *et al.*[20] have shown that in ECs the myosin phosphatase is inhibited by thrombin. Based on these observations, we suggest a model (see Figure 9) in which transient EC barrier failure is mediated by an activation of Ca^{2+}/CaM-dependent protein kinase I, whereas

prolongation of EC barrier dysfunction results from a sensitization of this process by a parallel activation of PTK and RhoA.

Under our experimental conditions, we could not find evidence for an important contribution of PKC to the thrombin-induced increase in endothelial permeability of human EC. Several lines of evidence make it unlikely that PKC plays a major role in the prolonged increase in human endothelial permeability induced by thrombin. First, incubation of human EC with PMA caused at moderate concentrations (1 and 10 nmol/L) a decrease in permeability, whereas the permeability increased only at very high concentrations, which give an extreme activation of PKC as compared to thrombin and histamine. Second, Ro31-8220 and Calphostin C, inhibitors of PKC, did not prevent the thrombin-induced increase in permeability. PMA and Ro31-8220 had also no effect on thrombin-stimulated MLC-phosphorylation. Third, histamine and thrombin induce a very similar rise in intracellular Ca^{2+} [38] and give a comparable activation of PKC in human EC.[39] For animal ECs the role of PKC in endothelial permeability has been established by several investigators.[17; 18] However, in HUVEC, the contribution of PKC could not be demonstrated unequivocally. Yamada et al. [40] showed that activation of PKC by PMA caused a decrease in endothelial permeability, whereas Bussolino *et al.*[41] found an increase in endothelial permeability by PKC activation. Garcia *et al.*[25] did not find any direct effect of PMA on human endothelial permeability and MLC-phosphorylation, whereas they found an increase of both by PMA in bovine ECs. Therefore, it seems that species differences are responsible for the discrepancies observed between the different studies.

In conclusion, we have demonstrated that in endothelial monolayers *in vitro*, which give a similar transient permeability response after exposure to histamine as expected from *in vivo* experiments,[1] thrombin induces a prolonged increase in permeability that involves protein tyrosine phosphorylation and RhoA activation. This mechanism appears comparable to the calcium sensitization observed in smooth muscle cells.[28] Future studies have to verify its occurrence in endothelial cells *in vivo* and elucidate whether a similar sensitization mechanism underlies the increased permeability that is enhanced by leukocytes and humoral factors circulating in patients with prolonged edema.

ACKNOWLEDGEMENTS

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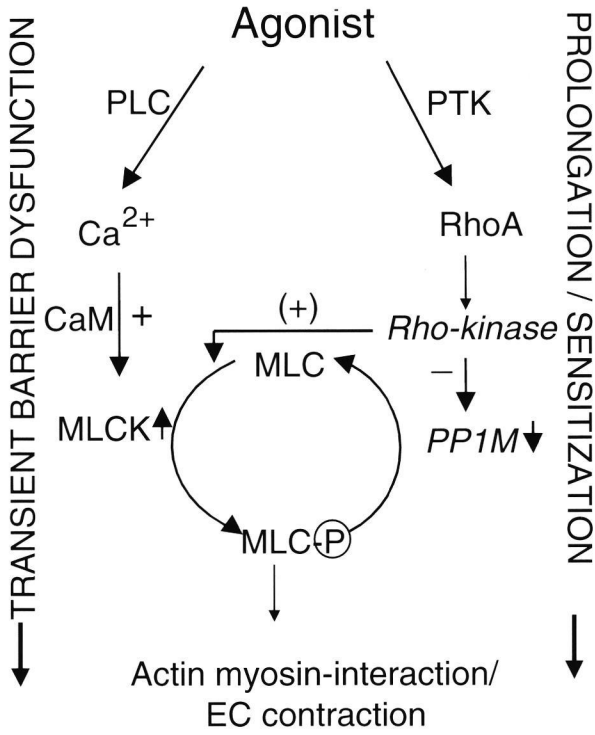

Figure 9. Proposed mechanisms affecting MLC phosphorylation that are involved in transient and prolonged EC barrier dysfunction. Elevation of intracellular Ca^{2+} by agonist exposure causes a transient increase of MLC phosphorylation by activation of a Ca^{2+}/CaM-dependent MLC kinase (MLCK). MLC phosphorylation can be prolonged by activation of RhoA in a PTK-dependent way. Rho kinase probably reduces the activity of myosin phosphatase type 1.[20] In addition to an effect of Rho kinase on the activity of myosin phosphatase 1, Rho kinase itself may act also as a MLC kinase.[36] It should be noted that RhoA also is involved in the organization of the actin cytoskeleton and in cell-cell and cell-matrix interactions. PLC indicates phospholipase C; PP1M, myosin phosphatase type 1; and MLC-P phosphorylated MLC.An interesting finding of this study is that although inhibition of PTKs by genistein lowers the basal MLC-phosphorylation status, it does not influence the relative increase in MLC-phosphorylation induced by histamine. First, this indicates that activation of the Ca^{2+}/CaM-dependent MLC kinase by histamine is not PTK-dependent under our experimental conditions. This is confirmed by the fact that inhibition of PTKs does not affect the histamine-induced EC contraction, as was measured by TEER. In addition, this finding suggests the existence of different pools of MLC. The relative increase in MLC phosphorylation that is caused by histamine is sufficient to induce contraction of genistein-treated ECs, although total MLC phosphorylation levels were lowered by inhibition of PTKs. Under these conditions MLC-phosphate levels did not exceed basal MLC-phosphate levels. This is of importance, because until now it was unclear how a generalized contraction of the endothelial cell could cause focal openings between the cells. One explanation may be that pools of MLCs located near the border of the cell are involved in the formation of the small gaps. These findings need further investigation.

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4

ROLE OF RHOA AND RHO KINASE IN LYSOPHOSPHATIDIC ACID-INDUCED ENDOTHELIAL BARRIER DYSFUNCTION

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ABSTRACT

In the present study, the role of the small GTPase Rho and Rho kinase in paracellular endothelial permeability was investigated *in vitro*. We have shown previously that, in addition to a rise in intracellular Ca^{2+}-ions, RhoA is involved in the prolonged thrombin-induced barrier dysfunction. To study the role of RhoA and Rho kinase more specifically, endothelial cells were stimulated with lyso-phosphatidic acid (LPA), a commonly used RhoA activator. LPA induced a 2-fold increase in horseradish peroxidase (HRP) passage that lasted for several hours, while thrombin induced a 5- to 10-fold increase. Comparable to the thrombin-induced barrier dysfunction, the LPA-induced barrier dysfunction was accompanied by a reorganization of the F-actin cytoskeleton. However, in contrast to thrombin, LPA induced only a transient increase in MLC phosphorylation, that returned to basal level within 10 minutes. In endothelial cells intracellular Ca^{2+}-levels were not elevated by LPA. Chelation of intracellular Ca^{2+}-ions by BAPTA did not prevent the LPA-induced HRP passage. Inhibition of RhoA by C3 transferase *of C. botulinum* inhibited the LPA-induced cytoskeletal changes. Inhibition of the Rho kinase by a specific inhibitor, Y-27632, completely prevented the LPA-induced increase in HRP passage. These data indicate that activation of RhoA is required to increase endothelial permeability, even without a change in intracellular Ca^{2+} concentration, and that Rho kinase is involved in the LPA-enhanced barrier dysfunction.

INTRODUCTION

The endothelium, which forms the inner lining of all blood vessels, is a highly selective barrier for blood constituents. Formation of gaps between endothelial cells (EC), for instance during inflammation, leads to extravasation of fluid and macromolecules and may cause life-threatening edema. This gap formation is based on an actin-nonmuscle myosin contraction process at the margins of the cell. Analogous to smooth muscle cell contraction, phosphorylation of the MLC by the Ca^{2+}/calmodulin-dependent kinase I, the classic MLC kinase, directs the actin-myosin-based contraction process in EC and is dependent on calcium ions and calmodulin.[1]

In the last few years it has become clear that MLC phosphorylation is a highly regulated process in which the small G-proteins play an eminent role. In HeLa cells overexpression of the p21-activated kinase (PAK)[2], an enzyme that is activated by the small G-proteins Cdc42 and Rac, reduces MLC kinase activity, whereas in EC PAK increases MLC phosphorylation.[3] The small G-protein RhoA, in addition to elevation of the $[Ca^{2+}]_i$, is involved in the prolonged endothelial barrier dysfunction and elevated MLC phosphorylation levels induced by thrombin.[4;5] Soluble factors from serum such as LPA and sphingosine-1-phosphate are throught to activate RhoA.[6;7] To address the question of whether activation of Rho by itself induces a prolonged endothelial barrier dysfunction we studied the effects of LPA on endothelial barrier function and investigated the mechanisms by which Rho can induce endothelial permeability.

The phospholipid LPA is known to be formed by and released from activated platelets and can be generated by action of secretory phospholipase A_2. Interestingly, Siess et al. recently have shown that mild oxidation of LDL also generates biologically active LPA, stimulating platelet activation and endothelial stress fiber formation.[8] LPA binds to a G-protein-coupled heptahelical receptor, which activates Rho via $G_{12/13}$.[9] When Rho is activated GDP is exchanged for GTP. Activation of Rho can be inhibited by the *C. botulinum* C3 transferase toxin, which specifically ADP-ribosylates Rho at Asn41.[10;11] In its GTP-bound form Rho interacts with several downstream targets, such as protein kinase N, Rho kinase, Rhotekin, Rhophilin, phosphatidylinositol 4-phosphate 5-kinase, citron and p140mDia (reviewed in[12-14]).

A good candidate for the Rho-induced cytoskeletal changes and cell contraction is Rho kinase. Rho kinase was shown to be involved in the formation of focal adhesion complexes[15;16] and to increase MLC phosphorylation by inhibition of myosin phosphatase activity[17] and possibly by direct MLC phosphorylation.[18] Uehata *et al.*[19] recently described a

synthetic pyridine-analog that inhibits the Rho kinase with high specifity compared to MLC kinase. This cell-permeant inhibitor, Y-27632, was able to prevent Rho-mediated stress fibers formation and smooth muscle contraction.

In the present study we investigated the effects of LPA-induced Rho activation on EC permeability, and used Y-27632 to demonstrate the involvement of Rho kinase in the LPA-enhanced endothelial permeability.

MATERIALS AND METHODS

Materials

Tissue culture plastics and Transwells (diameter 0.65 cm; pore size 3 μm) were obtained from Costar (Cambridge, MA); cell culture reagents as described previously.[4] Bovine thrombin was from Leo Pharmaceutical Products (Weesp, The Netherlands). Horseradish peroxidase (HRP) and anti-vinculin Ig were obtained from Sigma Chemical Company (St. Louis, MO). BAPTA-AM and rhodamine-phalloidin were from Molecular Probes (Eugene, OR). C3 transferase toxin was kindly provided by Dr A Ridley (Ludwig Institute, London). [^{32}P]-orthophosphoric acid and Tran^{35}S label were from ICN Pharmaceuticals, Inc. (Irvine, CA). Anti-platelet myosin Ig (non-muscle) was from Sanbio (Uden, The Netherlands); rabbit anti-mouse IgG-FITC from Dakopatts (Glostrup, Denmark). Y-27632 was supplied by Yoshitomi Pharmaceutical Industries (Saitama, Japan).

Cell culture and evaluation of barrier function

Human endothelial cells were isolated and cultured as previously indicated.[20;21] Barrier function was evaluated by the transfer of HRP across HUVEC monolayers grown on fibronectin-coated polycarbonate filters of the TranswellTM system.[4]

MLC phosphorylation

MLC phosphorylation was measured by double labeling technique. To that end, HUVEC were incubated for 24 hours with 150 μCi/mL Tran^{35}S-label and for 2 hours with 150 μCi/mL [^{32}P]orthophosphoric acid in phosphate-free buffer, prior to stimulation of the cells. Details have been given previously.[4]

Extraction and assay of intracellular cyclic AMP and calcium ion concentration

Intracellular cAMP levels were determined by radio-immunoassay (Amersham, Amersham, UK) as described previously.[4;20]

Intracellular calcium levels were determined by Fura-2 method as indicated previously.[20] As a positive control cells were permeabilized with 10 μmol/L Ionomycin and afterwards Ca^{2+}-ions were displaced from Fura-2 by incubation with 1 mM $MnCl_2$.

Immunocytochemistry

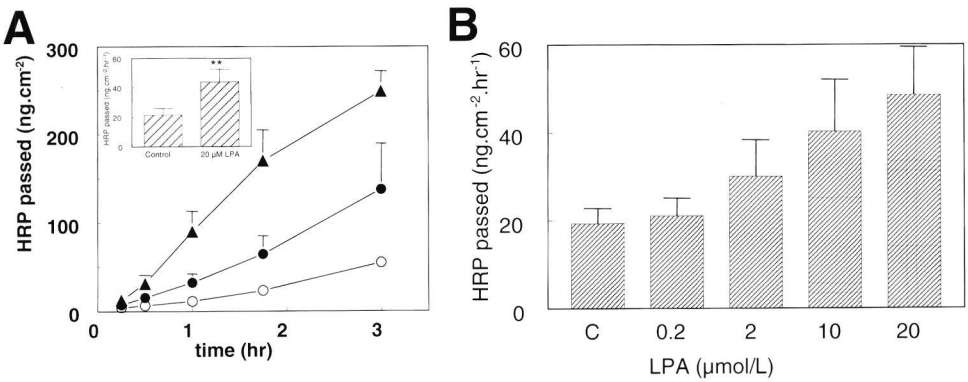
The presence of vinculin and F-actin was visualized by indirect immunofluorescence with mouse anti-vinculin antibody and by direct staining with rhodamine-phalloidin.

Statistical analysis

Data are reported as mean ± SD. Comparisons between more than two groups were made by one-way analysis of variance (ANOVA), followed by Bonferoni-adjusted chi-square test. Differences were considered significant at the $P < 0.05$ level.

RESULTS

LPA induces a prolonged decrease in endothelial barrier function



LPA induced an increase in endothelial permeability for peroxidase (HRP, figure 1, ● compared to basal HRP passage O). Comparably with the thrombin-induced increase in HRP passage (Figure 1A, ▲) the LPA-induced increase in permeability lasted for several hours. Although highly significant, the increase in HRP passage after stimulation by LPA was considerably lower compared to the 5- to 10-fold increase in permeability that is usually observed after stimulation by thrombin and just doubled after 1 hour (43.9 ± 8.7 $ng.cm^{-2}.hr^{-1}$ versus 21.4 ± 4.6 $ng.cm^{-2}.hr^{-1}$ in nonstimulated monolayers (mean ± SEM of 9 cultures in triplicate; $P<0.001$ Figure 1A, inset)). The effect of LPA on barrier function was concentration-dependent and started in the low micromolar range (Figure 1B).

Figure 1. Endothelial permeability is increased by LPA in a time- and concentration-dependent way.
A: Time-curve of the LPA-induced HRP passage across endothelial monolayers. Endothelial monolayers were stimulated with 20 µmol/L LPA (●), 1 U/mL thrombin (▲) or incubated without stimulation (O) and passage of HRP was measured as described in Materials & Methods. Values are the mean ± SEM of 2 different cultures in triplicate. Inset: HRP passage was doubled one hour after stimulation by LPA. Values are the mean ± SEM of 9 different cultures in triplicate. ** $P<0.001$ LPA-stimulated-cells versus control cells.
B: Concentration-curve of the LPA-induced HRP passage across endothelial monolayers. The HRP passage was determined 1 hour after sham treatment or exposure to the indicated concentrations of LPA. Values are the mean ± SD of 2 different cultures in triplicate.

The LPA-induced change in endothelial permeability was accompanied by changes in the F-actin cytoskeleton and localization of vinculin, a component of focal adhesion complexes. In confluent EC focal contact sites were hardly detectable (Figure 2B). Vinculin

appeared as a thin peripheral band and a diffuse cytoplasmic staining. F-actin filaments were organized in a cortical network (Figure 2A). Two minutes after activation with LPA vinculin appeared as a dense peripheral band and short F-actin filaments were formed (Figure 2C,D). After 10 minutes the F-actin content was further increased, thin fibers formed and small gaps between the cells became visible (Figure 2E). After 30 and 60 minutes the number of F-actin fibers was maximal (Figure 2G-J) and vinculin staining corresponded to the formation of focal adhesion complexes. After 120 minutes stress fibers disappeared completely, few focal attachment sites remained and no gaps could be observed (Figure 2K,L).

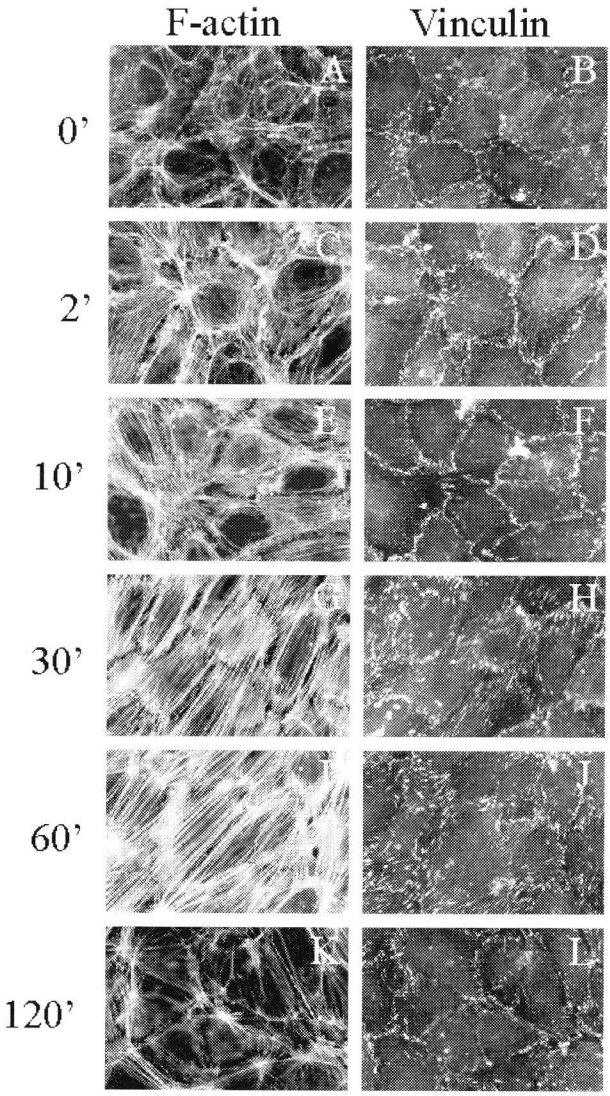


Figure 2. LPA induced changes in the F-actin cytoskeleton. Immunocytochemical staining of F-actin (A,C,E,G,I,K) and vinculin (B,D,F,H,J,L). EC were stimulated with 20 µmol/L LPA and fixed at the timepoints indicated. Cells were stained as described in Materials & Methods. Similar results were observed in 4 different cultures.LPA induces a transient phosphorylation of the MLCs

The parallel increase in F-actin filaments and enhanced endothelial permeability induced by LPA assumes an accompanying increase in MLC phosphorylation. LPA induced a transient increase in MLC phosphorylation, which was maximal 2 minutes after LPA addition and returned to basal levels within 10 minutes after LPA stimulation (Figure 3). In

contrast, after 10 minutes stimulation by thrombin MLC phosphorylation levels were still elevated (163 ± 17 % compared to basal levels (mean ± SEM of 3 cultures in duplicate)) and MLC phosphorylation remained elevated for at least 30 minutes as we have previously shown.[4]

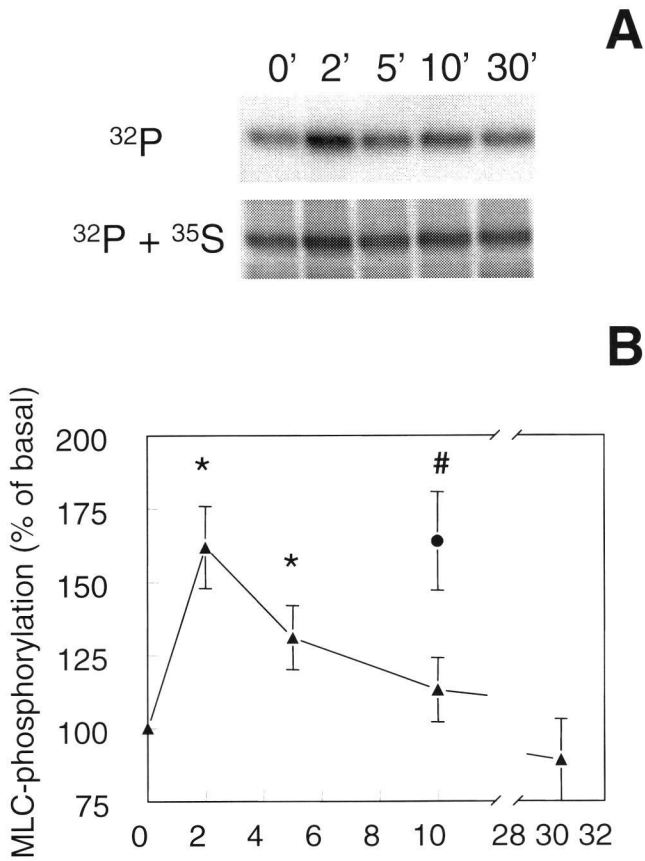


Figure 3. MLC phosphorylation is stimulated transiently by LPA. A, Autoradiograph of MLCs immunoprecipitated from cells under basal conditions (0 minutes) and 2, 5, 10, and 30 minutes after stimulation with 20 µmol/L LPA. Cells were labeled with ^{32}P and ^{35}S as described previously.[4] Top, An exposure in which a filter was present to block ^{35}S signal. Bottom, An exposure without filter. B, Quantification of MLC phosphorylation. LPA (▲) significantly increased MLC phosphorylation at 2 and 5 minutes. *P<0.05 LPA-stimulated versus nonstimulated cells. Values are the mean ± SD of 2 different cultures in duplicate. The level of ^{32}P incorporation into MLCs was calculated relative to the amount of ^{35}S incorporation into MLCs of the same sample. For comparison the effect of 1 U/mLthrombin (●) on MLC phosphorylation at 10 minutes is shown. #P<0.05 thrombin-stimulated versus nonstimulated cells.

Ca^{2+}-ions and cAMP are not involved in LPA-induced HRP passage

A transient elevation of $[Ca^{2+}]_i$ is involved in the thrombin-induced barrier dysfunction[22-24]. We investigated whether LPA also could induce a rise in intracellular Ca^{2+}-levels in HUVEC, as was shown in a variety of cell types[25] although these authors reported that several cell types lacked this Ca^{2+}-response. We found that LPA did not elevate the intracellular Ca^{2+}-concentration (Figure 4A), while thrombin induced a transient rise in intracellular Ca^{2+}-concentration in the same EC monolayers. To further exclude a role for Ca^{2+}-ions in the LPA-induced HRP passage, we pretreated EC with the intracellular Ca^{2+}-chelator BAPTA. Preincubation with BAPTA did not affect the LPA-induced HRP passage (Figure 4B), although it inhibited the thrombin-induced HRP passage by about 50 % in the same set of experiments (data not shown).[4]

LPA had no significant effect on cellular cAMP concentration at 2, 10 and 30 minutes after stimulation by LPA, while the adenylate cyclase activator forskolin increased cAMP in these cells (Figure 4C). This excludes the possibility that the LPA-increased endothelial permeability is caused by a reduction of intracellular cAMP levels.

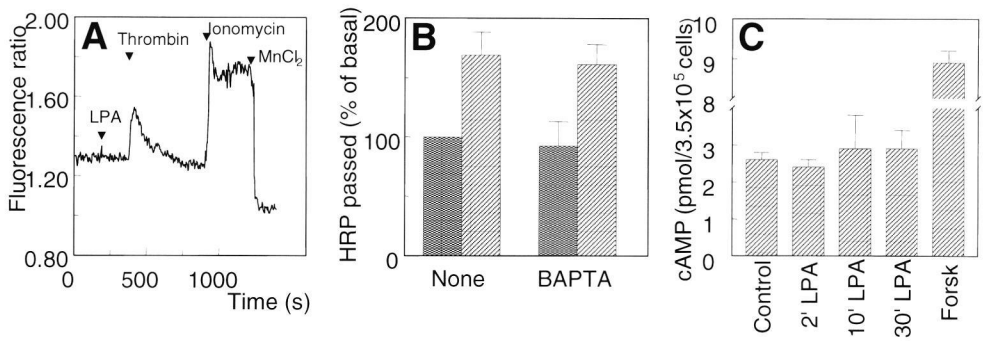


Figure 4. Intracellular calcium and cAMP levels are not involved in LPA-induced endothelial barrier dysfunction.

A: Representative tracing of changes in cell calcium in HUVEC exposed to LPA (20 µmol/L) and thrombin (1 U/mL). Cells were loaded with Fura2-AM and the intracellular calcium concentration was monitored as described in Materials & Methods. Similar results were obtained in 4 different cultures.

B: Effect of preincubation with BAPTA on basal (cross-hatched bars) and LPA-stimulated (hatched bars) HRP passage after one hour. Endothelial monolayers were preincubated for 1 hour with 3 µmol/L BAPTA. HRP passage is expressed as a percentage of basal HRP passage after 1 hour. Values are the mean ± SD of 2 different cultures in triplicate.

C: Effect of LPA (20 µmol/L) on cyclic AMP content. HUVEC were preincubated for 1 hour in Medium 199 + 1 % HSA and stimulated for the timepoints indicated with LPA (20 µmol/L) or vehicle. 15 min prior to stimulation cells were preincubated with IBMX (1 mM). Forskolin (10 µmol/L) was used as a positive control for cAMP elevation. Data are the mean ± SD of 3 different cultures in duplicate.

RhoA is involved in LPA-induced cytoskeletal reorganization

To verify that the effects of LPA on EC barrier function were mediated by RhoA, EC were pretreated with an inhibitor of RhoA, C3 transferase of *C. botulinum* and subsequently stained for vinculin and F-actin. Preincubation of the cells with the RhoA-inhibitor C3-transferase (5 µg/mL for 24 hours) had no effect on vinculin localization (Figure 5A,D), but the cortical F-actin network disappeared (Figure 5B,E). The F-actin that remained, appeared as a thin peripheral band that colocalized with vinculin (Figure 5C,F). The LPA-induced cytoskeletal changes (Figure 5G-I) were completely dependent on activation of RhoA, as preincubation with C3-transferase prevented the formation of focal adhesion complexes and the increase in F-actin filaments (Figure 5J-L). Preincubation with C3-transferase also completely prevented the LPA-induced HRP passage (data not shown). Thus, LPA induced a dramatic change in the endothelial F-actin cytoskeleton in a RhoA-dependent manner.

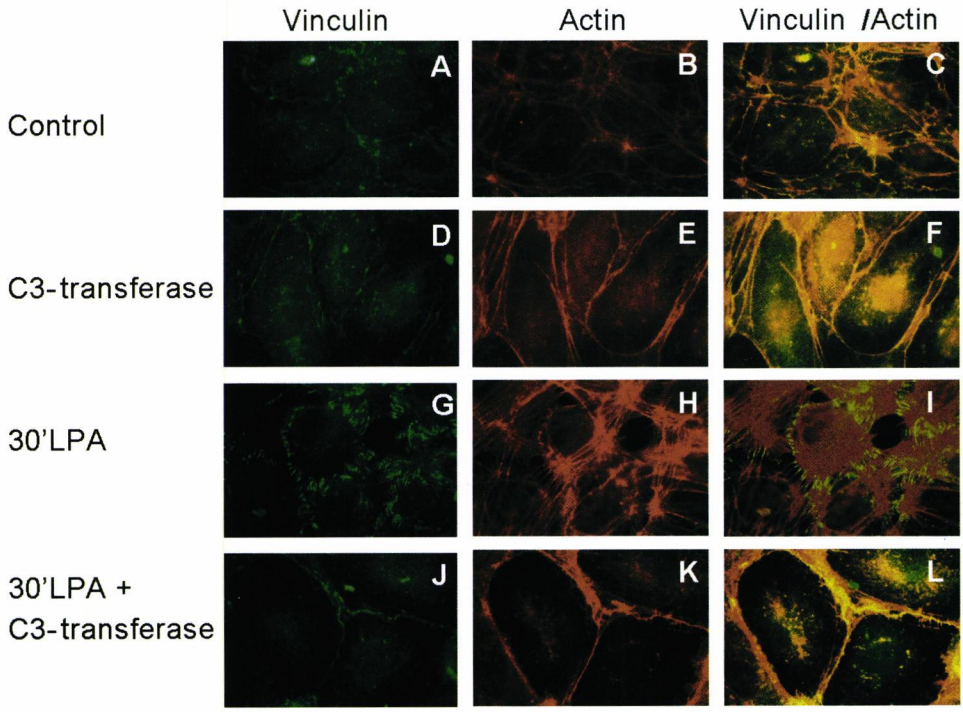


Figure 5. LPA-induced cytoskeletal reorganization is completely RhoA-dependent. Immunocytochemical staining of vinculin (A,D,G,J), F-actin (B,E,H,K) or both (C,F,I,L). HUVEC were preincubated for 24 hours with 5 µg/mL C3-transferase and stimulated with 20 µmol/L LPA. A,B,C: Basal condition. D,E,F,J,K,L: Cells were preincubated with C3-transferase. G,H,I,J,K,L: Cells were stimulated for 30 minutes with LPA. Similar results were observed in 3 different cultures.

Activation of Rho kinase is essential for LPA-enhanced permeability

Recent data pointed to a role of Rho kinase, one of the targets of RhoA, in the formation of stress fibers and cell contraction.[18] The Rho kinase inhibitor Y-27632 was used to study the role of Rho kinase in the LPA -induced endothelial barrier dysfunction. We first stained EC for F-actin, to verify that Rho kinase is involved in the LPA-induced EC cytoskeletal reorganization. Analogous to C3-transferase, Y-27632 completely prevented the LPA-induced F-actin polymerization (Figure 6A).

Then endothelial monolayers were preincubated for 1 hour with 10 µmol/L Y-27632. Preincubation with Y-27632 had no significant effect on basal permeability (Figure 6B), but completely prevented the LPA-induced increase in HRP passage. Thus, activation of Rho kinase by LPA is necessary for the proper formation of stress fibers and enhanced barrier dysfunction.

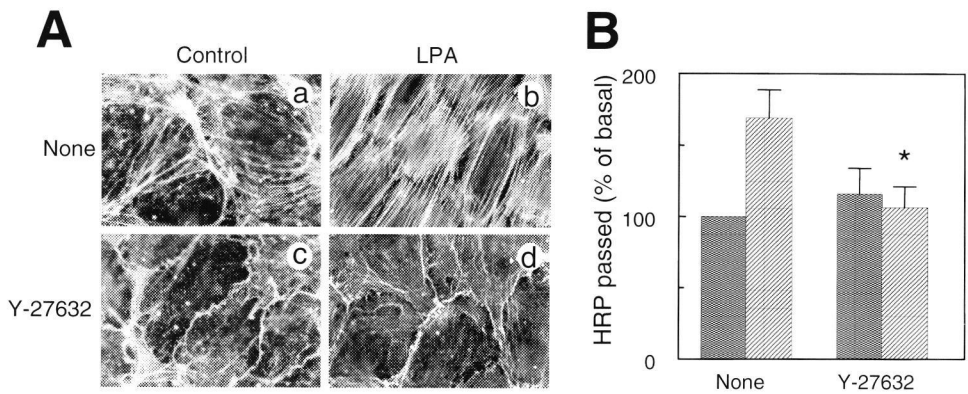


Figure 6. Effect of inhibition of Rho kinase on LPA-induced endothelial permeability.
A: Immunocytochemical staining of F-actin. EC were pretreated for 1 hour with 10 µmol/L Y-27632 (c, d) and stimulated for 30 minutes with 20 µmol/L LPA (b, d). Similar results were observed in 3 different cultures.
B: Effect of preincubation with Y-27632 on basal (cross-hatched bars) and LPA-stimulated (hatched bars) HRP passage after one hour. Endothelial monolayers were preincubated for 1 hour 10 µmol/L Y-27632. HRP passage is expressed as a percentage of basal HRP passage after 1 hour. Values are the mean ± SD of 2 different cultures in triplicate. *P<0.05 cells that were pretreated versus nonpretreated cells.

DISCUSSION

In the present study we show that activation of Rho by LPA is sufficient to induce a prolonged increase in endothelial permeability in the absence of a rise in intracellular

calcium. Furthermore, it was shown that LPA induces a transient increase in MLC phosphorylation and that the effect of LPA on barrier function was mediated by Rho kinase.

We have previously reported that, in addition to elevation of Ca^{2+}-ions, activation of Rho is necessary for the thrombin-induced MLC phosphorylation and enhanced endothelial permeability.[4] A model was postulated in which the prolonged effect of thrombin on endothelial barrier function was mediated by a Rho-dependent sensitization of a transient Ca^{2+}/CaM-dependent MLC phosphorylation, comparable to calcium sensitization known from smooth muscle cells.[26] In the present study the specific Rho activator LPA was used to study Rho-mediated mechanisms of endothelial permeability.

Several lines of evidence indicated that the LPA-induced endothelial permeability is Ca^{2+}-independent. First, after exposure to LPA no detectable rise in $[Ca^{2+}]_i$ could be observed in EC monolayers that responded to thrombin with respect to Ca^{2+}-mobilization. Second, chelation of intracellular Ca^{2+}-ions had no effect on the LPA-induced HRP passage. Thus, besides acting as a sensitisizer for Ca^{2+}-induced changes in thrombin-enhanced endothelial permeability[4], activation of RhoA is sufficient to induce endothelial permeability, even without a change in $[Ca^{2+}]_i$.

In fibroblasts it has been shown that LPA lowers cAMP levels, probably by coupling G_i-proteins to a LPA-receptor.[27] cAMP is known to improve endothelial barrier function.[22-24] However, LPA did not significantly lower cAMP levels in EC, excluding reduction of cAMP levels as the mechanism of LPA action.

Our finding that LPA induces endothelial permeability for macromolecules agrees with a previous report that showed an increase in tight junction permeability of pig brain capillary EC by LPA.[28] In a recent report by these investigators it was shown that LPA caused a serine/threonine dephosphorylation of the cadherin-associated adherens junctional proteins catenins p120 and p100 in a protein kinase C-independent way.[29] Form this data they suggested that LPA can actively regulate opening of adherens junctions. Other studies do not favour a role of Rho proteins in the regulation of adherens junctions in endothelial cells. Braga *et al.*[30] showed that EC are exceptional in this sense. They demonstrated that in contrast to other cell types Rho activity is not necessary for cadherin-based endothelial cell-cell interaction, and that VE-cadherin localization was insensitive to inhibition of either Rho or Rac. This fits with our finding that inhibition of RhoA and also Rho kinase did not decrease basal endothelial barrier function and that under these conditions inactivation of RhoA by C3 transferase did not disrupt the cortical F-actin band, although it reduced F-actin of EC. Furthermore, Essler *et al.*[5] showed that inhibition of Rho by C3-transferase did not prevent the thrombin-induced dissociation of catenins from the cytoskeleton. Wojciak-Stothard *et al.*[31]

showed that the Cdc42-, Rac- and Rho-dependent TNFα-induced stress fiber formation was also accompanied by an at least partly Cdc42-, Rac- and Rho-independent dispersion of VE-cadherin from intercellular junctions. Thus, at the moment there is no firm support for a role for Rho proteins in the direct regulation of adherens junction organization in EC. However, an indirect effect on proteins associated with adherens proteins cannot yet be excluded. This possibility still exists, because Rho kinase also acts on other proteins, such as adducin and members of the ERM family.[32] Recent data from Fukata *et al.* indicate that phosphorylation of adducin by Rho kinase activates the association of a F-actin-spectrin meshwork with the plasma membrane. Such mechanism may affect the organization of cytoskeleton anchoring with the plasma membrane and junction complexes.

In bovine aortic endothelial monolayers Alexander et al. observed an improvement of barrier function after stimulation with LPA.[33] These apparently contrasting results are not caused by tissue specific differences, as in our hands in human aortic endothelial monolayers LPA decreased barrier function, comparable to HUVEC monolayers (our unpublished observations, 1999), but may reflect species differences.

The LPA-induced endothelial barrier dysfunction requires Rho kinase activity, as was shown by inhibition of this kinase by Y-27632. With respect to endothelial permeability particular attention has been paid to its effect on MLC phosphorylation. Rho kinase can increase MLC phosphorylation by inhibiting the myosin phosphatase or by its capacity to phosphorylate the MLC by itself. The former appears the most likely mechanism of Rho kinase-induced MLC phosphorylation in EC, as it was shown that the myosin phosphatase is inhibited by thrombin in EC.[34;35]

A surprising finding of our study was that in contrast to the prolonged effect of LPA on endothelial barrier function, MLC phosphorylation was elevated only transiently by LPA. This suggests that a transient MLC phosphorylation is enough to induce a prolonged EC contraction comparable to the latch-bridge state in smooth muscle cells, where maintenance of contraction occurs despite MLC dephosphorylation.[36]

A transient MLC phosphorylation does not necessarily lead to a prolonged barrier dysfunction. Histamine was shown previously[4] to induce a very similar MLC phosphorylation, but it reduced barrier function in a transient and Rho-independent way. This suggests that either histamine activates a fast, unknown recovery process that is not activated by LPA or that LPA , in addition to MLC phosphorylation, does something more that results in a prolonged barrier dysfunction. It is plausible to suggest that the Rho kinase-induced focal adhesion formation[19] or other actions of the Rho kinase on the cytoskeleton[37] contribute to this prolongation.

The transient LPA-induced MLC phosphorylation assumes a transient activation of Rho. Indeed it was shown in adherent Swiss 3T3 cells that LPA induces a transient activation of RhoA.[38] Preliminary studies in our laboratory show that this is also the case in EC (our unpublished data). The transient MLC phosphorylation accords with the finding of Essler *et al.*[5], that showed that the myosin phosphatase was inhibited transiently by thrombin, an effect that could be reversed by C3-transferase.

The increase in MLC phosphorylation precedes the appearance of stress fibers and focal adhesions. This fits nicely with the model of Chrzanowska-Wodnicka and Burridge, who showed that the Rho-induced MLC phosphorylation and tension development are early events in the assembly of stress fibers in fibroblast cells, rather than a consequence of their formation.[39;40] The LPA-induced cytoskeletal changes were completely dependent on activation of Rho kinase.

Our data points to an important role of Rho and Rho kinase in the regulation of endothelial permeability. Future studies have to demonstrate if and when these factors are involved in altered endothelial barrier function *in vivo*. In large vessel EC, in particular in areas with altered shear forces stress fibers are found and Rho-mediated processes are likely to be involved. No information is presently available on microvascular EC *in vivo*. However, it should be noticed that Rho kinase plays a role in cell migration and that prolonged permeability might be a reflection of the altered behaviour of EC during cell migration and angiogenesis, which occur in wound healing and pathological conditions.

ACKNOWLEDGMENTS

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5

ACTIVATION OF RHOA BY THROMBIN IN ENDOTHELIAL HYPERPERMEABILITY

Role of Rho kinase and protein tyrosine kinases

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ABSTRACT

Endothelial cells (EC) actively regulate the extravasation of blood constituents. Upon stimulation by vasoactive agents and thrombin EC change their cytoskeletal architecture and small gaps are formed between neighbouring cells. These changes partly depend on a rise in intracellular Ca^{2+}-levels ($[Ca^{2+}]_i$) and activation of the Ca^{2+}/Calmodulin-dependent myosin light chain (MLC) kinase. In this study mechanisms that contribute to the thrombin-enhanced endothelial permeability were further investigated. We provide direct evidence that thrombin induces a rapid and transient activation of RhoA in human umbilical vein EC. Under the same conditions the activity of the related protein Rac was not affected. This was accompanied by an increase in MLC phosphorylation, the generation of F-actin stress fibers and a prolonged increase in endothelial permeability. Inhibition of the RhoA target Rho kinase with the specific inhibitor Y-27632 reduced all these effects markedly. In the presence of Y-27632 the thrombin-enhanced permeability was additionally reduced by chelation of $[Ca^{2+}]_i$ by BAPTA. These data indicate that RhoA/Rho kinase and Ca^{2+} represent two pathways that act on MLC phosphorylation. In addition, the protein tyrosine kinase inhibitor genistein reduced thrombin-induced endothelial permeability without affecting activation of RhoA by thrombin. Our data support a model of thrombin-induced endothelial permeability that is regulated by three cellular signal transduction pathways.

INTRODUCTION

The endothelium is the main barrier for blood constituents and actively regulates the extravasation of blood components to the surrounding tissues. Formation of minute gaps between EC, for instance during inflammation, leads to extravasation of fluid and macromolecules, and may cause life-threatening edema. Analogous to smooth muscle cell contraction, phosphorylation of the MLC by the Ca^{2+}/calmodulin-dependent kinase I, the classic MLC kinase, directs the actin-myosin-based contraction process in EC and is dependent on calcium ions and calmodulin.[1] In addition to a Ca^{2+}/calmodulin-dependent transient increase in permeability induced by histamine and substance P[2], thrombin induces a prolonged disturbance of endothelial barrier function.[3] This is associated with a reorientation of the F-actin cytoskeleton, a prolonged MLC phosphorylation and the formation of intercellular gaps.[4] In contrast to smooth muscle cells, MLC phosphorylation in EC *in vivo* does not result in a general contraction of the cells, but a contraction process does occur at the margins of the cell.[2]

In the last few years it has become clear that MLC phosphorylation is a highly regulated process in which small G-proteins of the Rho family play a crucial role. Most attention has been paid to the small GTPase RhoA. In smooth muscle cells activation of RhoA results in 'calcium sensitization', *i.e.*, independently of changes in $[Ca^{2+}]_i$, MLC phosphate levels increase by inhibition of the smooth muscle myosin phosphatase, resulting in force generation.[5-7] Other proteins of the Rho family of small GTPases also could be involved in MLC phosphorylation. Activation of the p21-activated kinase (Pak), an enzyme that is activated by the small G-proteins Cdc42 and Rac, was shown to affect MLC phosphorylation.[8;9] In HeLa cells overexpression of Pak reduces MLC kinase activity.[9] In contrast, in EC Pak appears to increase MLC phosphorylation by being an MLC kinase itself.[8;10]

It is a matter of debate which Rho-like small GTPases are involved in endothelial permeability. In human umbilical vein EC the thrombin-induced endothelial hyperpermeability was reduced by C3 transferase from *C. botulinum*, a specific inhibitor of Rho.[4;11] C3 transferase also reduced the thrombin-induced MLC phosphorylation.[4;11;12] Several investigators have doubted a role of RhoA in thrombin-enhanced permeability[13], or suggested that Rac participates in cytoskeletal remodeling by thrombin in EC.[14]

In this study, we investigated whether thrombin induces an activation of RhoA and Rac using two newly developed assays for activation of RhoA[15;16] and Rac.[17;18] Protein tyrosine kinases (PTK) have been implicated in intracellular signalling in thrombin-enhanced barrier dysfunction by many investigators.[4;12;19;20] Because a genistein-sensitive PTK has been reported to act upstream of RhoA in lysophosphatidic acid-stimulated EC,[21] we subsequently studied whether PTK also are required for activation of RhoA by thrombin. Furthermore, we evaluated the involvement of Rho kinase, a downstream target of RhoA, in the thrombin-enhanced endothelial permeability and MLC phosphorylation using the Rho kinase inhibitor Y-27632.[22]

MATERIALS AND METHODS

Materials

Cell culture reagents were obtained as described previously.[4] Bovine thrombin was from Leo Pharmaceutical Products (Weesp, The Netherlands). Horseradish peroxidase (HRP) was obtained from Sigma Chemical Company (St. Louis, MO). BAPTA-AM and rhodamine-phalloidin were from Molecular Probes (Eugene, OR). Genistein was from Alexis Inc. (San Diego, CA). Y-27632 was supplied by Yoshitomi Pharmaceutical Industries (Saitama, Japan). [^{32}P]-orthophosphoric acid and Tran^{35}S label were from ICN Pharmaceuticals, Inc. (Irvine, CA). Antibody against Rho kinase was kindly provided by Dr L Lim (Institute for Molecular and Cell Biology, Singapore).[23] Antibody against RhoA was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against Rac was from Transduction Laboratories (Lexington, KY). Anti-platelet myosin Ig (non-muscle) was from Sanbio (Uden, The Netherlands). Secondary antibodies were from Dakopatts (Glostrup, Denmark).

Cell culture and evaluation of barrier function

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as previously indicated.[24] Barrier function was evaluated by the transfer of HRP (42-44 kD) across HUVEC monolayers grown on fibronectin-coated polycarbonate filters of the TranswellTM system.[4]

MLC phosphorylation

MLC phosphorylation was measured by a double labeling technique. To that end, HUVEC were incubated for 24 hours with 150 µCi/mL Tran^{35}S-label and for 2 hours with 150 µCi/mL [^{32}P]orthophosphoric acid in phosphate-free buffer, prior to stimulation of the cells. Details have been given previously.[4]

Rho-activity assay

Rhotekin-binding assays were essentially performed as described with some minor modifications.[15;16] Briefly, 20 cm^2 confluent HUVEC were preincubated for one hour in Medium 199 + 1 % HSA. Cells were stimulated and lysed. Lysates were cleared by centrifugation and incubated with bacterially produced GST-RBD (where RBD stands for the Rho-binding domain of Rhotekin and GST for gluthathione-S-transferase) immobilized on glutathione-coupled Sepharose beads for 30 min at 4^{0}C. Beads were washed, eluted in Laemmli sample buffer and analyzed by Western blotting using a rabbit polyclonal anti-RhoA antibody in a 1:200 dilution.

Rac-activity assay

Pak-binding assays were performed as described with some minor modifications.[17;18] Briefly, 20 cm^2 confluent HUVEC were preincubated for one hour in Medium 199 + 1 % HSA. Cells were stimulated and lysed. Lysates were cleared by centrifugation and incubated with bacterially produced GST-PAK-CD (where CD stands for Cdc42/rac1 Interactive Binding domain, *i.e.*, the GTPase binding domain) immobilized on glutathione-coupled Sepharose beads for 30 min at 4^0C. Beads were washed, eluted in Laemmli sample buffer and analyzed by Western blotting using a mouse monoclonal anti-Rac1 antibody in a 1:2000 dilution.

Detection of Rho kinase by immunoblotting

Detection of Rho kinase by immunoblotting was performed as indicated by Lim *et al.*[23]

Immunocytochemistry

The presence of F-actin was visualized by direct staining with rhodamine-phalloidin.

Statistical analysis

Data are reported as mean ± SEM. Comparisons between more than two groups were made by one-way analysis of variance (ANOVA), followed by the Bonferoni-adjusted chi-square test. Comparisons of time curves of two groups were made using repeated measures ANOVA. Individual groups comparisons were done using a Student's t-test for post hoc comparisons of the means. Differences were considered significant at $P < 0.05$.

RESULTS

Activation of RhoA but not Rac by thrombin

To evaluate whether thrombin directly activates RhoA and Rac in EC, we used 2 newly developed assays for RhoA and Rac activity. The assay for RhoA activity is based on binding of activated RhoA (RhoA-GTP) to a GST-Rhotekin fusion protein,[15;18] which is reflected in the pull-down fraction of Figure 1 (upper panel). Under control conditions hardly any RhoA-GTP could be detected. The amount of RhoA-GTP in the pull-down fraction was greatly enhanced 30 seconds after stimulation with 1 U/mL thrombin, and diminished after 5 minutes. The overall amount of RhoA did not change (Figure 1, lower panel). In contrast, the activity of Rac, which was measured by use of a GST-Pak fusion protein[18], did not change or even slightly decreased after thrombin stimulation (Figure 1 middle panel). With the same reagents activation of Rac by bradykinin in rat PC12 cells was demonstrated previously (see ref [18]). Thus, thrombin rapidly and transiently activates RhoA but not Rac in HUVEC.

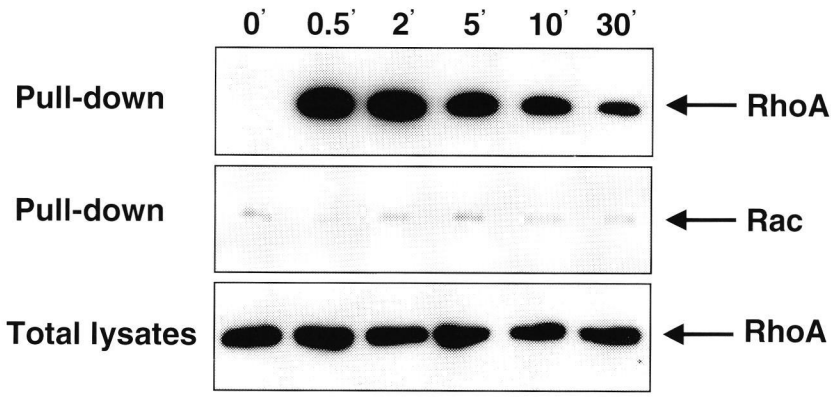

Figure 1. Thrombin transiently activates RhoA, and has no effect on Rac in human endothelial cells.
Upper panel: Immunoblot showing activation of RhoA after exposure of various timepoints of HUVEC to 1 U/mL thrombin. Cell lysates were incubated with GST-RBD beads as described in Materials and Methods, the beads washed and the bound protein analyzed by Western blotting using an antibody specific for RhoA.
Middle panel: Immunoblot showing, that thrombin has no effect on Rac in the same samples. Cell lysates were incubated with GST-PAK beads. The bound protein was analyzed by Western blotting using an antibody specific for Rac.
Lower panel: Before performing the GST pull-down, 1/40 of each sample was analyzed on Western blot using an antibody specific for RhoA, showing that equal amounts of protein were present in all samples. Similar results were obtained in 3 independent EC cultures.

Rho kinase is involved in the thrombin-enhanced permeability

To investigate whether RhoA acts in endothelial permeability via Rho kinase the cell-permeant Rho kinase inhibitor Y-27632 was used, at a high concentration of 10 µmol/L that was shown to completely inhibit Rho kinase.[22] We first checked by Western blotting whether Rho kinase was present in EC and found one single band at the expected molecular mass of 160 kD in HUVEC (Figure 2A inset) and other types of human macro- and microvascular EC (glomerular and foreskin microvascular EC, iliac artery and vein EC, aorta EC; data not shown).

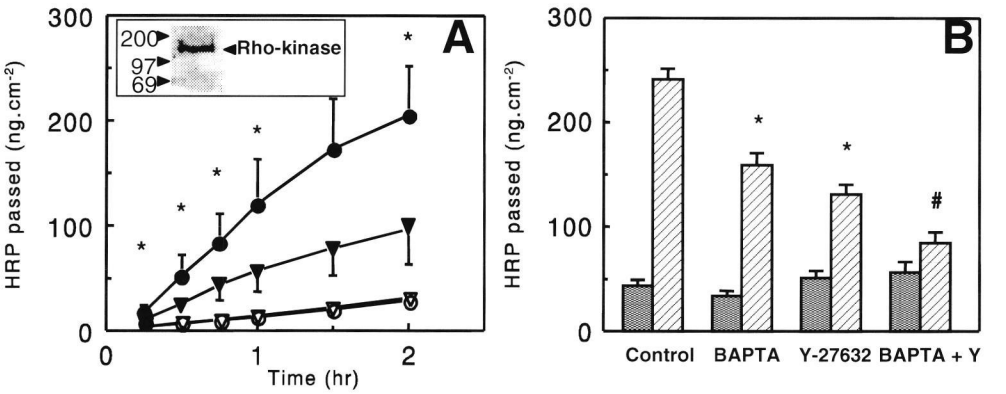

Figure 2. Involvement of Rho kinase in thrombin-enhanced endothelial permeability.

A: Effect of the Rho kinase inhibitor Y-27632 on the passage of HRP through HUVEC monolayers under basal conditions (O,▽) and after exposure to 1 U/mL thrombin (●,▼). Cells were preincubated for 1 hour in Medium 199 + 1 % HSA with 10 µmol/L Y-27632 (▽,▼) or without addition (O,●) and HRP passage was measured as described in Materials and Methods. Values are the mean ± SEM of 9 cultures in three independent experiments. The interaction between time and Y-27632 treatment was significant (P=0.000) for thrombin-stimulated cells. Treatment with Y-27632 resulted in lower HRP passage compared with no treatment. *P<0.05, Y-27632-pretreated versus nonpretreated cells that were stimulated with thrombin.

Inset: Detection of Rho kinase by Western blot in HUVEC extracts. The estimated molecular mass of the protein was 160 kDa.

B: Effect of BAPTA and Y-27632 on basal (cross-hatched bars) and thrombin-induced HRP passage (hatched bars) across HUVEC monolayers. Cells were pretreated for 1 hour with 3 µmol/L BAPTA, 10 µmol/L Y-27632 or both. HRP passage was measured 2 hours after sham treatment or exposure to 1 U/mL thrombin. Values are the mean ± SD of 6 cultures in two experiments. *P<0.05, cells pretreated with BAPTA or Y-27632 versus nonpretreated cells. #P<0.05, cells pretreated with a combination of BAPTA and Y-27632 versus pretreatment with each compound alone.

The Rho kinase inhibitor Y-27632 was used to study the role of Rho kinase in the thrombin-induced endothelial barrier dysfunction. Preincubation for 1 hour with 10 µmol/L Y-27632[22] had no effect on basal permeability (Figure 2A), but significantly attenuated the thrombin-enhanced endothelial permeability, as evidenced by an decreased passage of the markermolecule HRP through the endothelial monolayer. This attenuation was partial, even when Y-27632 was used at a higher concentration (up to 100 µmol/L was tested, which had the same effect as 10 µmol/L) or when its preincubation time was prolonged to 3 hours (data not shown).

Inhibition of Rho kinase by Y-27632, which was maximal at 10 µmol/L, together with chelation of intracellular Ca^{2+}-ions by BAPTA (Figure 2B) nearly completely blocked the thrombin-enhanced HRP passage, suggesting that RhoA/Rho kinase- and Ca^{2+}- dependent processes act on EC permeability by separate pathways.

Inhibition of Rho kinase reduces MLC phosphorylation and cytoskeletal reorganization

Alterations in endothelial permeability are accompanied by actin nonmuscle myosin-interaction, which is regulated by MLC phosphorylation. MLC phosphorylation, as determined by ^{32}P incorporation in MLC, was increased upon activation of EC by thrombin in agreement with previous observations (Figure 3).[3;4;11;25] Preincubation with the Rho kinase inhibitor Y-27632 significantly reduced MLC phosphorylation, both under basal and thrombin-stimulated conditions (Figure 3). Formation of both thrombin-induced F-actin stress fibers and small gaps between neighbouring EC was largely prevented by Y-27632 (Figure 4). Thus, activation of Rho kinase by thrombin is involved in the thrombin-induced MLC phosphorylation and is required for the proper formation of stress fibers and small gaps.

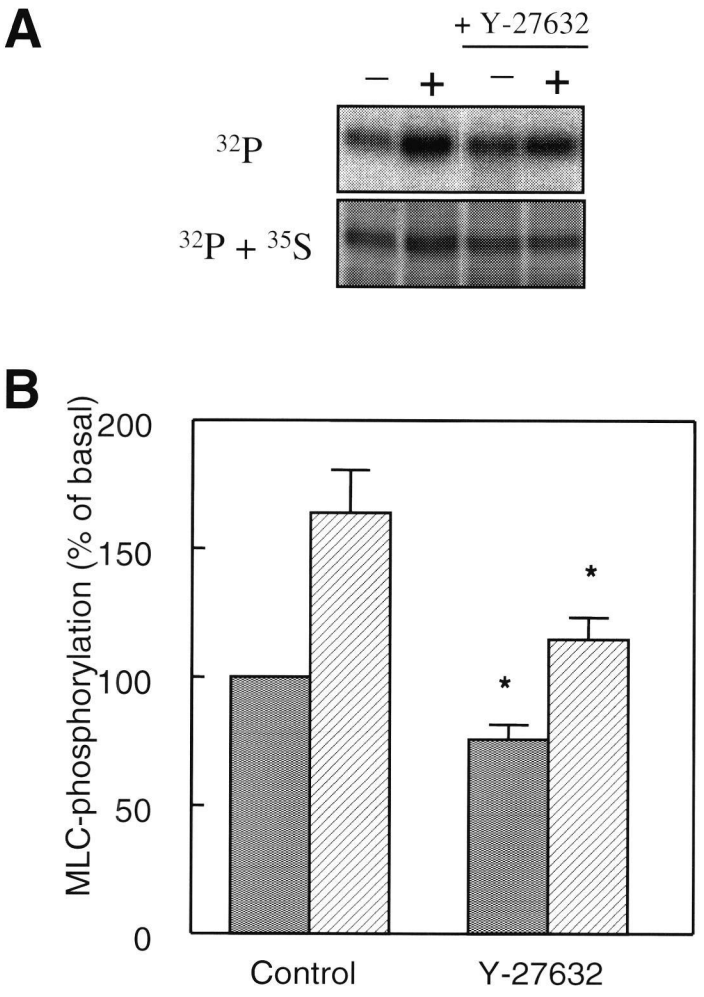

Figure 3. MLC phosphorylation is inhibited by Y-27632.

A, Autoradiograph of MLCs immunoprecipitated from cells under basal conditions (-) and 10 minutes after stimulation with 1 U/mL thrombin (+). Cells were labeled with ^{32}P and ^{35}S as described previously,[4] and preincubated with 10 µmol/L Y-27632 for one hour. MLC phosphorylation was measured as described in Materials and Methods. Top, An exposure in which a filter was present to block ^{35}S signal. Bottom, An exposure without filter.

B, Quantification of MLC phosphorylation. Effect of 10 µmol/L Y-27632 on basal (cross-hatched bars) and thrombin-enhanced (hatched bars) MLC phosphorylation. The level of ^{32}P incorporation into MLCs was calculated relative to the amount of ^{35}S incorporation into MLCs of the same sample, as was described previously.[4] Values are the mean ± SEM of 3 different cultures in duplicate. #P<0.05 Y-27632-pretreated versus nonpretreated cells.

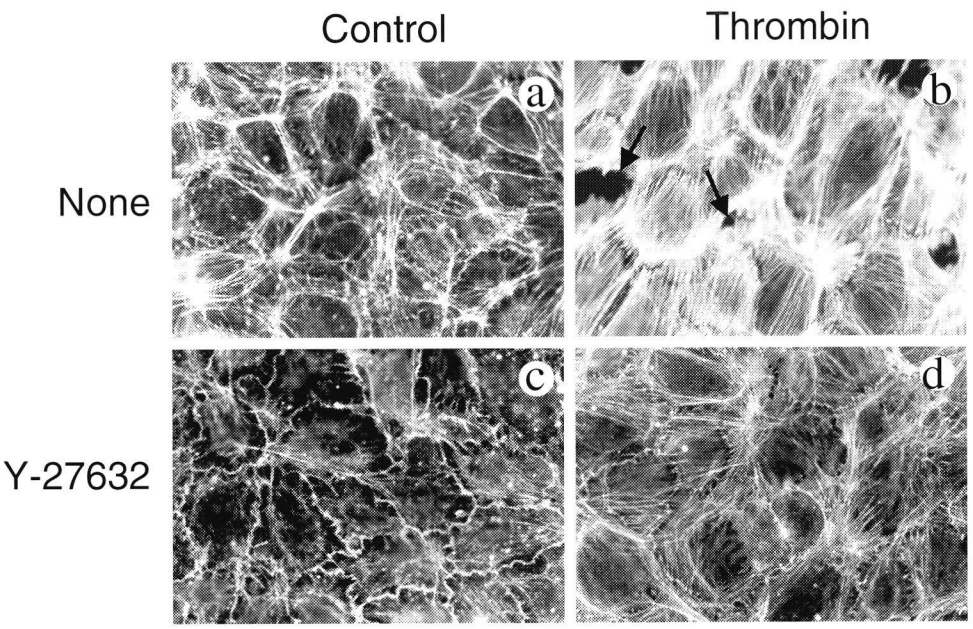

Figure 4. Effect of Y-27632 on F-actin cytoskeleton.
Immunocytochemical staining of F-actin in HUVEC grown on glass cover slips. EC were preincubated for 1 hour in Medium 199 + 1 % HSA in the absence (a, b) or the presence of 10 µmol/L Y-27632 (c, d) and stimulated for 30 minutes with 1 U/mL thrombin (b, d). Gaps between the cells are indicated with black arrows in b. Similar results were observed in 3 different cultures.

Inhibition of PTK reduces thrombin-induced permeability, but not RhoA activation

To investigate whether the PTK involved in endothelial permeability act upstream of Rho activation, EC were preincubated for 1 hour with 30 µg/mL of the PTK inhibitor genistein. This dose of genistein is a maximally effective dose for inhibiting endothelial barrier dysfunction.[4] Genistein did not affect the thrombin-induced activation of RhoA (Figure 5 inset). In parallel cultures genistein inhibited the thrombin-enhanced passage of HRP through the endothelial monolayer (Figure 5). This indicates that the genistein-sensitive PTK, which are involved in endothelial barrier dysfunction, do not act upstream of RhoA activation.

Subsequently, endothelial monolayers were coincubated with genistein and Y-27632 to test whether PTK act downstream of Rho kinase in inducing endothelial barrier dysfunction. The inhibition of the thrombin-induced HRP passage by genistein was additional to the inhibition by Y-27632 (Figure 5). This indicates that a PTK-dependent proces also acts on a RhoA/Rho kinase-independent pathway, that affects EC permeability.

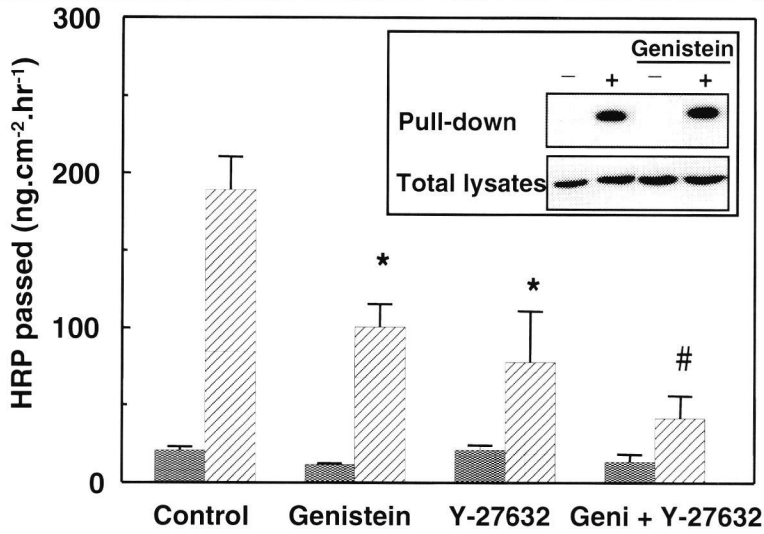

Figure 5. Effect of genistein and Y-27632 on basal (cross-hatched) and thrombin-enhanced permeability (hatched bars). Endothelial monolayers were pretreated for 1 hour with 30 µg/mL genistein, 10 µmol/L Y-27632, or both. The HRP passage was determined 1 hour after sham treatment or exposure to 1 U/mL thrombin. Values are the mean ± SD of 6 cultures in two experiments. *P<0.05, cells pretreated with genistein or Y-27632 versus nonpretreated cells. #P<0.05, cells pretreated with a combination of genistein and Y-27632 versus pretreatment with each compound alone.

Inset: Genistein has no effect on thrombin-induced activation of RhoA. HUVEC were preincubated with 30 µg/mL genistein for 1 hour and stimulated with 1 U/mL thrombin for 1 minute. A Western blot of the pull-down of activated RhoA and 1/40 of total RhoA is shown.

DISCUSSION

In the present study we provide direct evidence that thrombin potently activates RhoA in EC, but does not affect the activity of Rac. Activity of the PTK involved in endothelial hyperpermeability was not required for the thrombin-induced activation of RhoA. Furthermore, it was shown that thrombin increases endothelial permeability via Rho kinase independent from a rise in $[Ca^{2+}]_i$ and that PTK and Rho kinase have additive effects on endothelial hyperpermeability.

To our knowledge we provide the first direct demonstration that RhoA is activated by thrombin in confluent endothelial monolayers. Its rapid onset was comparable to the rise in $[Ca^{2+}]_i$. However, RhoA activation extended beyond the transient (5 minutes) rise in $[Ca^{2+}]_i$; some RhoA-GTP was still detectable after 30 minutes. Under basal conditions hardly any active RhoA was detectable, suggesting a minor role of RhoA in basal barrier integrity.

Previous studies indirectly suggested the involvement of RhoA in endothelial permeability, largely based on the use of C3 transferase. This toxin penetrates the cell with difficulty and requires long preincubation times. Thus, it may interfere with gene regulation.[26] Other investigators have used that Toxin B, which inhibits both RhoA, Rac and Cdc42. Inactivation of both RhoA, Rac and Cdc42 disrupts the endothelial barrier[27], whereas inactivation of RhoA alone enhances endothelial barrier function.[13] It is therefore likely, that Toxin B exerts its disruptive effect not via RhoA, but acts either via Rac or Cdc42. Under our experimental conditions thrombin activated RhoA, but not Rac. This makes the involvement of the Rac target Pak, which is able to induce endothelial retraction,[8] in thrombin-enhanced permeability less likely.

In addition to RhoA thrombin-enhanced endothelial permeability requires Ca^{2+}-ions. Similarly as previously found with the Rho inhibitor C3 transferase inhibition of Rho kinase inhibited the increased permeability, but only partly at the maximally effective dose of Y-27632. In combination with the chelation of Ca^{2+}-ions Y-27632 almost completely prevented the thrombin-induced HRP passage. This does not only indicate that both Ca^{2+}-ions and the RhoA/Rho kinase pathway are necessary for the full thrombin response, but also that RhoA/Rho kinase signaling and Ca^{2+}-dependent processes additionally contribute barrier dysfunction.

MLC phosphorylation plays a pivotal role in initiating actin-myosin interaction and in the development of barrier dysfunction.[4] Here, we extend our previous observation on RhoA[4] and show that Rho kinase contributes to MLC phosphorylation in EC. This is comparable with the situation in smooth muscle cells[28] and blood platelets,[29-31] where Rho kinase has recently been shown to be involved in MLC phosphorylation. Rho kinase can increase MLC phosphorylation by inhibiting the myosin phosphatase or by its ability to phosphorylate the MLC itself. The former appears the most likely mechanism of Rho kinase-induced MLC phosphorylation in EC, as it was shown that in EC the myosin phosphatase is inhibited by thrombin,[32;33] and that the inhibition of Rho by C3 transferase prevents this inhibition of the phosphatase.[11] In addition to an effect on MLC phosphorylation, Rho kinase also phosphorylates other proteins. Several of these proteins are involved in stress fiber formation and may act on EC barrier function, including LIM kinase,[34] adducin and members of the ERM (Ezrin/Radixin/Moesin) family.[35] Their involvement in endothelial permeability remains to be demonstrated.

We have shown previously, that in addition to a rise in intracellular Ca^{2+}-ions, PTK are involved in the thrombin-enhanced endothelial barrier dysfunction.[4] Other investigators demonstrated that a genistein-sensitive PTK acts upstream of lysophosphatidic acid-induced

RhoA activation in EC.[21] The present study shows that inhibition of PTK with genistein does not influence activation of RhoA by thrombin, indicating that PTK do not act upstream of the activation of RhoA in thrombin-enhanced endothelial permeability. This suggests differences between activation of RhoA by either lysophosphatidic acid or thrombin, which may be the involvement of G_{13}. G_{13} is involved in the activation of RhoA by lysophosphatidic acid, but has not been demonstrated to be involved in thrombin-induced signal transduction.[36]

Inhibition of PTK with genistein reduced the thrombin-enhanced barrier dysfunction in addition to the effect of Y-27632. This indicates that PTK and Rho kinase at least act by separate pathways. Interestingly, this and our previous study show that chelation of Ca^{2+} by BAPTA acts in addition to the inhibition of both PTK and Rho kinase. This suggests that the Ca^{2+}-, RhoA-, and PTK-mediated pathways induced by thrombin reflect separate pathways, that all converge in increased permeability. For the RhoA- and Ca^{2+}-dependent pathways this convergence point is probably the MLC phosphorylation (compare Figure 6, the left part).

Inhibition of PTK may act on endothelial permeability by various mechanisms. PTK inhibitors attenuate agonist-induced increases in $[Ca^{2+}]_i$.[37;38] In accordance with this, Garcia *et al.* recently reported that activity of an endothelial-specific MLC kinase is regulated by tyrosine phosphorylation in a RhoA-dependent manner.[12] However, additional mechanisms must exist, as PTK are involved in endothelial barrier dysfunction independent of changes in $[Ca^{2+}]_i$[4] and RhoA signaling (present study). A probable mechanism is the destabilization or disruption of adherens junctions by tyrosine phosphorylation of junctional proteins. The agonist-induced disruption of adherens junctions is accompanied by tyrosine phosphorylation of VE-cadherin and the associated β- and γ-catenin, and this results in the dissociation of VE-cadherin/catenins.[39] Disintegration of junctional complexes and the actin-nonmuscle myosin interaction in the periphery of EC may thus act in concerted prolonged thrombin-induced endothelial permeability (Figure 6).

Our data point to an important role for RhoA and Rho kinase in the regulation of endothelial permeability. Future studies have to demonstrate if and when these factors are involved in altered endothelial barrier function *in vivo*. In large vessel EC, in particular in areas with altered shear forces stress fibers are found and Rho-mediated processes are likely to be involved. The recent finding of Essler *et al.*[40] that mildly oxidized LDL activates Rho/Rho kinase signalling in endothelial cells, suggest the involvement of RhoA and Rho kinase in vascular leakage during the development of atherosclerosis. No information is presently available on microvascular EC *in vivo*. However, it should be noticed that Rho kinase plays a role in cell migration and that prolonged permeability might be a reflection of

the altered behavior of EC during cell migration and angiogenesis, which occur in wound healing and pathological conditions

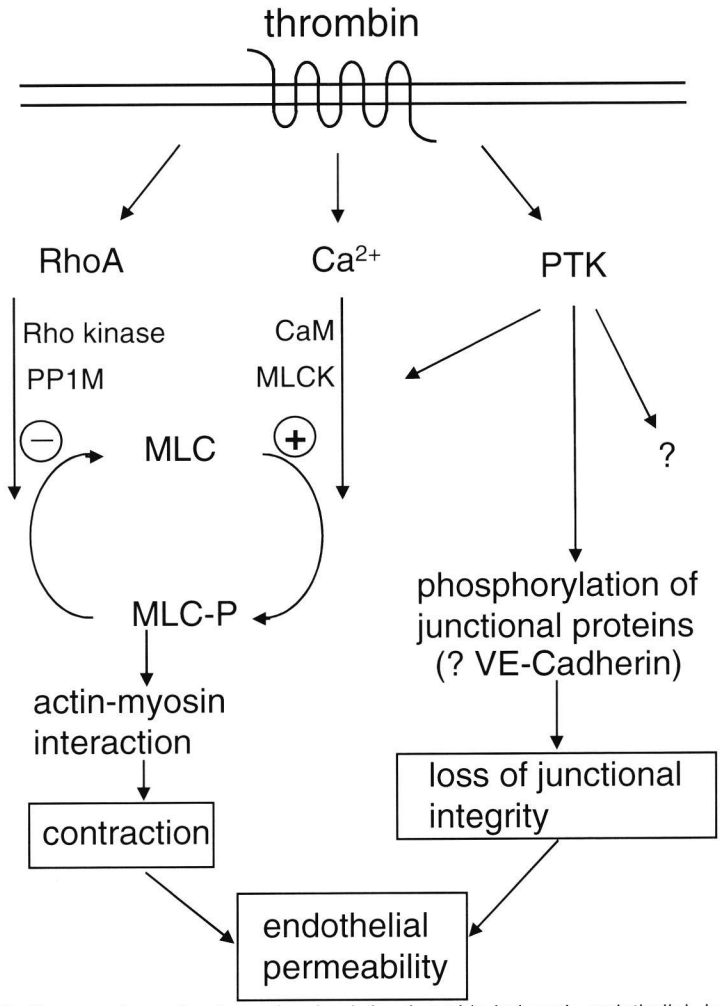

Figure 6. Proposed mechanisms involved in thrombin-induced endothelial barrier dysfunction. Thrombin induces Ca^{2+}-dependent activation of MLC kinase as well as RhoA/Rho kinase-dependent inhibition of the myosin phosphatase. Both facilitate MLC phosphorylation, which causes an increased actin-myosin interaction, and a contraction process at the margins of the cells, which finally results in a disturbed endothelial barrier function. In addition to Ca^{2+}- and RhoA-dependent processes, thrombin induced PTK activities act on phosphorylation of junctional proteins, as VE-Cadherin and its associated catenins, which results in disruption of intercellular junctions.[39] CaM indicates calmodulin; MLC myosin light chain; MLCK MLC kinase; MLC-P, phosphorylated MLC; PP1M, myosin phosphatase type 1; and PTK, protein tyrosine kinase.

ACKNOWLEDGMENTS

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6

SIMVASTATIN IMPROVES DISTURBED ENDOTHELIAL BARRIER FUNCTION

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ABSTRACT

Recent clinical trials have firmly established that inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (statins) reduce the risk of acute coronary events. These effects of statins cannot be fully explained by their lipid-lowering potential. Improved endothelial function may contribute to the positive effects of statin treatment. In the present study we report that simvastatin treatment is effective in reducing endothelial barrier dysfunction, which is associated with the development of atherosclerosis. Treatment of human umbilical vein endothelial cells (HUVEC) for 24 hours with 5 μmol/L simvastatin reduced the thrombin-induced endothelial barrier dysfunction *in vitro* by 55 ± 3 %, as assessed by the passage of peroxidase through HUVEC monolayers. Similar effects were found on the thrombin-induced passage of ^{125}I-LDL through human aortic endothelial cell monolayers. This reduction in barrier dysfunction by simvastatin was both dose- and time-dependent, and was accompanied by a reduction in the thrombin-induced formation of stress fibers and focal adhesions. Simvastatin treatment had no effect on cellular cAMP levels. In Watanabe-heritable hyperlipidemic rabbits, treatment for 1 month with 15 mg/kg simvastatin reduced vascular leakage, both in the thoracic and abdominal part of the aorta, as evidenced by Evans blue dye exclusion test. The decrease in endothelial permeability was not accompanied by a reduction of Oil red O-stainable atherosclerotic lesions. This data shows that simvastatin improves disturbed endothelial barrier function both *in vitro* and *in vivo*, and further support the beneficial effects of simvastatin in acute coronary events by other mechanisms than its lipid lowering effect.

INTRODUCTION

Recent clinical trials have shown that inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (statins), the rate-limiting enzyme of cholesterol synthesis in the liver, are effective in the prevention of acute coronary events. Further analysis of these studies suggests that the benefits of statin therapy cannot be fully explained on the basis of reductions in plasma cholesterol levels.[1;2] Recent studies demonstrated that treatment of hypercholesterolemic patients with statins improved endothelial function and vasomotion.[3;4] These effects of statins on endothelial function can be fast in onset as an improvement of endothelial function was reported within one month of simvastatin therapy in patients with moderate serum cholesterol.[5]

Experimental atherosclerosis in hypercholesterolemia models is associated with changes in endothelial integrity (reviewed in [6]). Additionally, during the development of atherosclerosis, the permeability of the vessel wall for LDL increases (reviewed in [7]). Since gross endothelial loss probably only occurs very late in atherosclerosis development, dysfunction of the intact endothelial layer provides a more likely explanation for the increase in permeability.

The endothelial actin cytoskeleton is very important in maintaining the structural integrity of the endothelium.[8] The junction-associated actin filament system forms a dense peripheral band of F-actin and is the most prominent assembly in the majority of endothelial cells.[9] Activation of the endothelium by inflammatory mediators results in a contraction process at the margins of the cell. Small gaps between neighbouring cells are formed, increasing the permeability of the endothelium. *In vitro* these changes in permeability are accompanied by the formation of long F-actin filaments or stress fibers.[10] *In vivo* these stress fibers are found in areas of altered flow. The function of these stress fibers, which span the entire cell, is not precisely known at the moment, but they could have contractile properties.[11]

In this study we investigated whether simvastatin could improve disturbed endothelial barrier function, both in an *in vitro* and in an *in vivo* model of endothelial perturbation.

MATERIALS & METHODS

Materials

Tissue culture plastics and Transwells (diameter 0.65 cm; pore size 3 µm) were obtained from Corning Costar (Cambridge, MA); cell culture reagents as described previously.[10] Bovine thrombin was from Leo Pharmaceutical Products (Weesp, The Netherlands). Horseradish peroxidase (HRP), Evans blue, Oil red O, and anti-vinculin Ig were obtained from Sigma Chemical Company (St. Louis, MO). Rhodamine-phalloidin was from Molecular Probes (Eugene, OR). Rabbit anti-mouse IgG-FITC

from Dakopatts (Glostrup, Denmark). Simvastatin-lactone used *in vitro* studies was from Merck, Sharp & Dohm (Rahway, NJ, USA), and *in vivo* was from Sankyo (Tokyo, Japan). ^{125}I-iodine was purchased from Amersham (Amersham, UK).

Cell culture and evaluation of barrier function *in vitro*

Human endothelial cells were isolated and cultured as described previously.[12] Confluent HUVEC were used after 1 passage; confluent human aortic EC after 4 passages. Barrier function was evaluated by the transfer of HRP or ^{125}I-LDL across highly confluent EC monolayers grown on fibronectin-coated polycarbonate filters of the TranswellTM system.[10;13;14]

Preparation and iodonation of Low Density Lipoproteins

LDL was isolated from fresh serum prepared from the blood of healthy volunteers by gradient ultracentrifugation[15] and labeled with ^{125}I-iodine as previously described.[14]

Extraction and assay of intracellular cyclic AMP levels

Intracellular cAMP levels were determined by radio-immunoassay from Amersham (Amersham, UK) as described previously.[10;12]

Immunocytochemistry

The presence of vinculin and F-actin was visualized by indirect immunofluorescence with mouse anti-vinculin antibody and by direct staining with rhodamine-phalloidin using a confocal laser scan microscope (type TCS 4D, Leica Heidelberg, Heidelberg, Germany). Overlaying of pictures was accomplished using Photo-Paint software (version 6.00: 1995; Corel Co., Ottawa, Canada).

Animals

16 months-old Watanabe-heritable hyperlipidemic WHHL rabbits were obtained from the Oriental Yeast Company, Tokyo, Japan. They were housed individually and fed on standard rabbit chow *ad libitum*, with water supplied *ad libitum*. For four weeks prior to sacrifice, the animals received simvastatin-lactone (15.0 mg/kg body weight) daily, in addition to the standard rabbit chow.

Cholesterol measurement

Total serum cholesterol was determined using a commercially available enzymatic kit (Roche Diagnostics, Mannheim, Germany).

Evaluation of vascular leakage and atherosclerosis

Animals were sedated with Hypnorm (0.4 ml/kg i.m.), mildly anticoagulated with heparin (500 U/rabbit i.v.) and euthanised with i.v. Nembutal (2.5 ml/kg). The aorta was rapidly removed, cleaned from the adjacent tissue and perfused with Medium 199 buffered with Hanks' salts, 100 IU/mL penicillin, 100 µg/mL streptomycin and 1% serum albumin to remove blood cells and then incubated with Evans blue in Medium 199 (0.3% [wt/vol]) for 5 minutes. Aortas were incubated with Medium 199 to remove unbound Evans blue and fixed with 3.7% buffered formaldehyde. Aortas were opened longitudinally and photographed en face.

Subsequently, the extent of atherosclerosis was assessed by staining the Evans blue-treated aortic segments with Oil red O and evaluated.

Statistical analysis

Data are reported as mean ± SEM. Comparisons between more than two groups were made by one-way analysis of variance (ANOVA), followed by Bonferoni-adjusted chi-square test. Comparisons of time curves of two groups were made using repeated measures analysis of variance (MANOVA) and individual group comparisons were done using a Student's t-test for post hoc comparisons of the means. Differences were considered significant at the $P < 0.05$ level.

RESULTS

Simvastatin induces a change in cell morphology

Confluent human umbilical vein and human aortic endothelial monolayers were incubated for 24 hours in Medium 199 containing 5 µmol/L simvastatin in the presence of serum to evaluate the effect on cell morphology (Figure 1). Simvastatin-treated EC remained tightly confluent, but cells were slightly elongated –which was most easily visible in aortic monolayers- with a condensed cytoplasm around the nucleus.

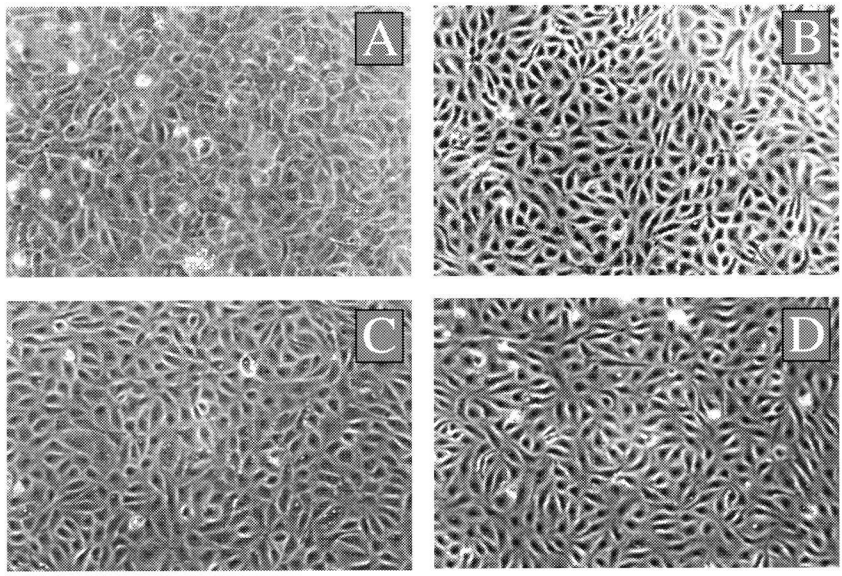

Figure 1. Effect of simvastatin on morphology of human umbilical vein endothelial cells of passage number 1 (A, B) and human aortic endothelial cells of passage number 4 (C, D). Phase-contrast micrograph of control cells (A, C) and cells preincubated for 24 hours with 5 µM simvastatin (B, D).

Effects of simvastatin on *in vitro* endothelial barrier function

To test whether simvastatin influences endothelial barrier function, HUVEC were grown to confluence on porous filters and the passage of a marker protein, HRP (42-44 kD) was measured. Pretreatment with 5 µmol/L simvastatin for 24 hours slightly increased HRP passage. However, the thrombin-induced HRP passage was significantly decreased from 30 minutes onwards (Figure 2A). Simvastatin pretreatment inhibited thrombin-induced HRP

passage by 55 ± 3 % during a one-hour incubation period (mean ± SEM, 5 different cultures in triplicate). The reduction was concentration-dependent (Figure 2B), and was maximal at 5 µmol/L simvastatin. Higher concentrations were not used as they affected basal EC barrier function. Coincubation with mevalonate fully abolished the effect of simvastatin on cell morphology (not shown) and thrombin-induced HRP passage (Figure 2B), indicating that simvastatin reduced the thrombin-induced HRP passage by inhibition of HMG-CoA reductase. Simvastatin inhibited the thrombin-induced HRP passage in a time-dependent manner (Figure 2C). This inhibition was maximal after 8 hours of simvastatin pretreatment and continued for at least 24 hours. Thus, simvastatin reduced the thrombin-induced HRP passage across HUVEC monolayers in a time- and concentration-dependent manner.

As atherosclerosis is associated with an enhanced passage of LDL across the aortic endothelium and an increase in accumulation of LDL in the aortic wall,[7] we preincubated human aortic EC monolayers with simvastatin and measured its effect on (thrombin-induced) ^{125}I-LDL passage. Simvastatin slightly increased basal aortic endothelial permeability, but attenuated thrombin-enhanced LDL passage and was maximally effective at 2 µmol/L (Figure 2D).

Simvastatin does not affect cellular cAMP and eNOS levels

Elevation of intracellular cAMP levels is known to improve endothelial barrier function.[16] Therefore, we tested whether simvastatin increased intracellular cAMP levels. When HUVEC were preincubated with 5 µmol/L simvastatin for 24 hours cAMP concentration remained unaltered both under basal conditions (2.6 ± 0.2 and 1.9 ± 0.4 pmol/3.5x10^5 cells in control and simvastatin-pretreated cells) and after 2 minutes' stimulation of the cells with 1 U/mL thrombin (2.5 ± 0.8 and 2.1 ± 0.4 pmol/3.5x10^5 cells) or after 30 minutes' stimulation (2.6 ± 0.1 and 2.1 ± 0.2 pmol/3.5x10^5 cells, respectively; 3 experiments). As a positive control cells were pretreated for 15 minutes with 10 µM forskolin with raised intracellular cAMP concentration to 9.0 ± 0.4 pmol/3.5x10^5 cells. This excludes elevation of cAMP as the mechanism of action.

It has been reported that statins may increase the cellular amount of eNOS.[17-19] We have previously shown that NO is a negative feedback regulator of the thrombin-induced barrier dysfunction.[20] However, pretreatment of human umbilical vein and aortic endothelial monolayers with 5 µmol/L simvastatin for 24 hours had no effect on eNOS protein expression as revealed by Western blotting (data not shown).

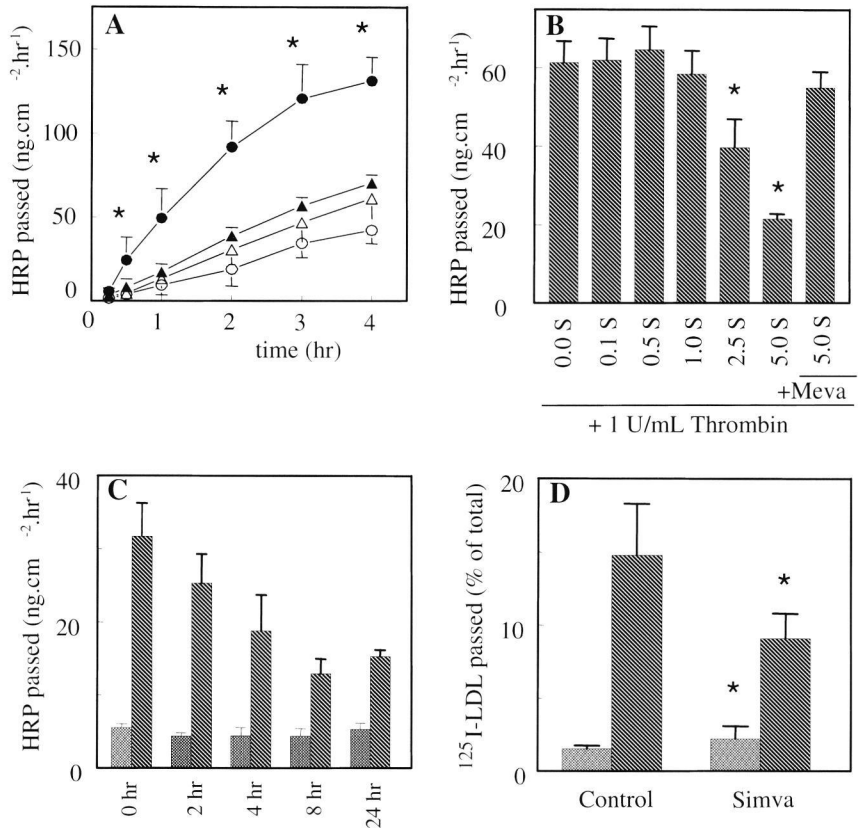

Figure 2. Effect of simvastatin on human endothelial barrier function.

A: Effect of simvastatin on the passage of HRP through human umbilical vein endothelial monolayers under basal conditions (O,Δ) and after exposure to 1 U/mL thrombin (●, ▲). Cells were preincubated for 24 hours with 5 µmol/L simvastatin (Δ, ▲) or without addition (O,●). Values are the mean ± SEM of 2 different cultures in triplicate. The interaction between time and simvastatin treatment was significant (P=0.000) for thrombin-stimulated cells. Treatment with simvastatin resulted in lower HRP passage compared with no treatment. Differences were significant from 30 minutes onward. *P<0.05, simvastatin-pretreated versus nonpretreated cells that were stimulated with thrombin. HRP passage was measured as described in Materials & Methods.

B: Simvastatin decreased the thrombin-induced HRP passage through human umbilical vein endothelial monolayers in a dose-dependent manner. Cells were pretreated for 24 hours with simvastatin (0.1, 0.5 1.0, 2.5, or 5.0 µmol/L) in the presence or absence of 100 µmol/L mevalonic acid. HRP passage was measured 1 hour after exposure to 1 U/mL thrombin. Values are the mean ± SEM of 2 different cultures in triplicate. There was a significant difference between HRP passage of the various thrombin-stimulated groups (P=0.000). *P<0.05, cells that were pretreated with simvastatin versus nonpretreated cells.

C: Simvastatin decreases the thrombin-induced HRP passage in a time-dependent manner. HUVEC monolayers were pretreated for the indicated time periods and HRP passage was measured under basal conditions (filled bars) and after 1 hour after stimulation with 1 U/mL thrombin (hatched bars). Values are the mean ± SD of a representative experiment out of 3 experiments.
D: Simvastatin decreases the thrombin-induced ^{125}I-LDL passage through human aortic endothelial monolayers. Cells were pretreated for 24 hours with 2 µmol/L simvastatin. HRP passage was measured 1 hour after exposure to 1 U/mL thrombin (hatched bars) or sham treatment (cross-hatched bars). Values are the mean ± SEM of 3 independent experiments in triplicate. *P<0.05, cells that were pretreated with simvastatin versus non-pretreated cells.

Effect of simvastatin on localization of vinculin and F-actin of basal and thrombin-stimulated monolayers

The actin cytoskeleton of EC is very important in maintaining the structural integrity of the endothelium.[8] To see whether simvastatin influenced endothelial permeability by changes in the cytoskeleton, HUVEC monolayers were stained for vinculin, a marker for focal adhesion formation, and for F-actin. Under control conditions hardly any vinculin staining was observed, indicating the resting state of the monolayers. Control cells were characterized by cortical F-actin microfilaments (Figure 3A-C). Thrombin induced a dramatic increase in the formation of focal adhesions and stress fibers (Figure 3G-I). Many small gaps between the cells were observed. Pretreatment with simvastatin decreased basal F-actin filaments and only a thin peripheral rim of F-actin remained (Figure 3D-F). Formation of focal adhesions by thrombin was reduced by simvastatin pretreatment, as was the formation of stress fibers and of gaps (Figure 3J-L). Thus, simvastatin to a large extent prevented the thrombin-induced changes of the EC cytoskeleton.

Simvastatin treatment reduces vascular leakage *in vivo*

To test whether simvastatin could improve endothelial barrier function *in vivo* Watanabe rabbits were treated with 15 mg/kg body weigth simvastatin over 4 weeks. Simvastatin induced a slight, non-significant, reduction in plasma cholesterol (from 10.2 ± 2.7 before to 8.5 ± 2.1 mmol/L after simvastatin treatment; n=3, p=0.30), but cholesterol levels remained elevated after this relatively short treatment period. After sacrifice of the animals, endothelial barrier function of the thoracic and abdominal aorta was assessed *ex vivo* by determining the penetration of the Evans blue-albumin complex in the vessel wall. In the thoracic aortas of control animals an intense blue staining was observed (Figure 4A). Treatment with simvastatin reduced Evans blue staining (Figure 4A). Similar results were obtained in the abdominal aorta of the same rabbits (data not shown).

To exclude that the decrease in endothelial permeability by simvastatin treatment was due to a reduction in the extent of atherosclerotic plaques, the same aorta segments were subsequently stained with Oil red O. Aortas either from control or simvastatin-treated animals were both severely atherosclerotic, with no visible changes in lipid accumulation after simvastatin treatment for 4 weeks (Figure 4B). As a control, umbilical veins were used, which showed no Oil red O staining (data not shown). It is of interest to note that in the control animals a strong blue staining was observed in the areas of low lipid accumulation (Figure 4A, white arrow). This extravasation is not likely to be caused by damage of the vessel wall, because in aorta areas of simvastatin-treated animals with a comparable low degree of lipid accumulation (Figure 4B, yellow arrow), a relatively small amount of Evans blue-albumin complex was observed (Figure 4A, yellow arrow). This exclusion of Evans blue dye indicates the presence of an intact endothelial barrier. It is likely that the accumulated lipids interfered with maximal accumulation of Evans blue. Thus, simvastatin improved vascular barrier function in Watanabe rabbits already at a time point that no obvious changes in the extent of atherosclerotic plaques were observed.

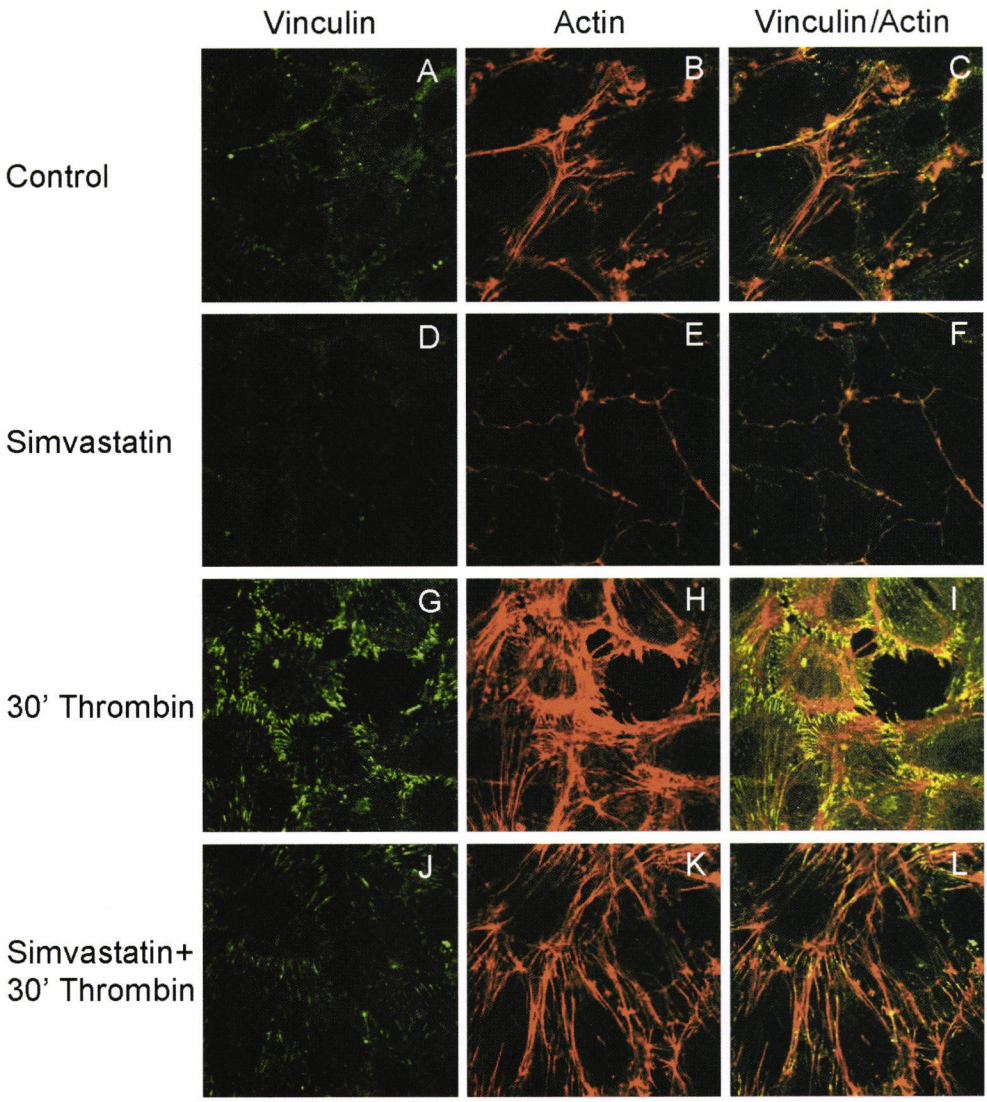

Figure 3. Thrombin-induced cytoskeletal reorganization is reduced by simvastatin. Immunocytochemical staining of vinculin (A, D, G, J), F-actin (B, E, H, K) or both (C, F, I, L). HUVECs were preincubated for 24 hours with 5 µmol/L simvastatin and stimulated with 1 U/mL thrombin. Cells were stained as described in Materials & Methods. A, B, C: Basal condition. D, E, F, J, K, L: Cells preincubated with simvastatin. G, H, I, J, K, L: Cells stimulated for 30 minutes with thrombin. Similar results were observed in 3 different cultures.

A
Evans blue
Control
Simvastatin
1
2
3
B
Oil Red O
Control
Simvastatin

Figure 4. Simvastatin reduces vascular leakage in Watanabe rabbits without a regression of the presence of atherosclerotic plaques.
A: Groups of 3 Watanabe rabbits were treated for 4 weeks with 15 mg/kg body weight simvastatin or placebo and were sacrified. Aortas were carefully preparated and Evans blue dye exclusion test was performed as indicated in Materials & Methods.
B: Afterwards the same aortas were stained with Oil red O as described in Materials & Methods.
Arrows of the same colour indicate corresponding regions in Evans Blue- and Oil Red O-stained aortas, where less visible atherosclerosis had developed. For colour figure see included CD-ROM.

DISCUSSION

The major finding of this study is that simvastatin treatment reduced endothelial barrier dysfunction both in an established *in vitro* model for endothelial permeability consisting of human endothelial cells grown on porous filters as *ex vivo* in aorta segments of Watanabe rabbits, which have an increased endothelial permeability.

Accumulation of LDL in the arterial wall is one of the hallmarks of the development of atherosclerosis. The focal nature of plaque formation is remarkably similar to the focal occurrence of vascular leakage sites.[7] In laboratory animals the regional variation in the arterial wall permeability predicts the pattern of experimental atherosclerosis. It is not known at the moment whether this association reflects a causal relationship. Our finding that simvastatin treatment, which decreases the development of atherosclerosis, reduces enhanced permeability, supports the idea that an increased permeability to LDL increases the risk of atherosclerosis and that a reduction in endothelial permeability, in addition to a lowering in plasma LDL concentration, may assist in the prevention of atherosclerosis progression.

We have previously shown that NO improves endothelial barrier *in vitro* and acts as a negative feedback regulator of the thrombin-induced barrier dysfunction.[20] It has been reported that statins increase eNOS expression on the post-transcriptional level in cultured EC, so statins may improve barrier function by increasing NO production.[17-19] However, during the relatively short period of our *in vitro* experiments we did not find any upregulation of eNOS protein expression, which excluded this as the mechanism of improvement of barrier function. Nor did simvastatin have any effect on intracellular cAMP concentration, which is also known to improve endothelial barrier function.[16]

Simvastatin reduced F-actin staining in control HUVEC monolayers, in accordance with Koch *et al.*,[21] who found a decrease in F-content in NIH 3T3 cells by lovastatin treatment, while G-actin content remained unaltered. Furthermore, simvastatin largely

reduced the formation of stress fibers induced by thrombin. This finding is of interest as prominent stress fibers are found in endothelial cells *in vivo* in areas prone to the development of atherosclerosis.

Several authors have shown that statins may affect the F-actin cytoskeleton through inactivation of Rho proteins.[21] Lovastatin prevented the LPA-induced translocation of RhoA, shape changes and cell contraction in neuronal cells by inhibition of isoprenylation of RhoA.[22] An effect of statins on Rho proteins in vascular smooth muscle and endothelial cells also have recently been indicated.[17;23] We and others have previously shown that RhoA is involved in the thrombin-induced endothelial barrier dysfunction *in vitro*.[10;24] To our knowledge no *in vivo* data are yet available on the role of activation of RhoA in vascular leakage.

Improvement of endothelial integrity by simvastatin *in vivo* was probably not due to a reduction of atherosclerotic lesions. A short regime of 4 weeks of simvastatin treatment was chosen, which had only a moderate effect on plasma cholesterol levels (17% reduction). No visible plaque regression was detectable in the rabbit aortas after Oil red O staining.

Statin treatment also improves clinical outcome following coronary stent implantation and reduces recurrence rates.[25] It appeared that the beneficial effects of statin therapy are not related to its lipid-lowering effects. Van Beusekom *et al.* have previously shown that stenting especially decreases long-term vascular integrity with respect to permeability and endothelial proliferation, and is associated with distinct morphologic characteristics: endothelial retraction, expression of surface folds and the adhesion of leukocytes, even after 12 weeks with a complete endothelial covering.[26] Besides inhibition of smooth muscle cell proliferation, improvement of endothelial barrier function may contribute to improved clinical outcome of post-stent patients receiving statin treatment.

We observed that highly confluent cells were necessary for simvastatin having a positive effect on disturbed barrier function *in vitro* (our unpublished observations). One explanation for this finding may be that, because statins are known to inhibit cell proliferation, nonconfluent monolayers were not able to reach confluency in the presence of simvastatin.[27;28] Furthermore, in vascular smooth muscle cells statins are known to induce apoptosis at high concentration.[23] Taken together, this means that precaution has to be taken to use statins when endothelial cells are in a proliferative state, e.g. in wound healing.

Treatment of patients suffering from prolonged edema has been less successfull with current therapies up till now. Although β-adrenergic agents have been shown to be effective

in acutely induced vascular leakage, when administered in capillary leakage syndrome desensitization to β-adrenergics occurs after one day.[29;30] Future studies are necessary to investigate whether statin treatment could reduce vascular leakage under these conditions.

In conclusion, we found that simvastatin treament reduces endothelial permeability and that this decrease in permeability was accompanied by a decrease in cell F-actin content. These findings may have implications for the treatment of patients with a high risk of developing atherosclerosis, and with implanted stents, and possibly in patients with capillary leakage syndrome.

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7

ROLE OF RHO KINASE IN VEGF-INDUCED ENDOTHELIAL CELL MIGRATION AND ANGIOGENESIS IN VITRO

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ABSTRACT

Vascular Endothelial Growth Factor (VEGF) potently stimulates endothelial cell (EC) migration and angiogenesis. Reorganization of the F-actin cytoskeleton and cell-matrix adhesion is essential in these processes. In the present study we investigated whether Rho kinase is involved in the VEGF-induced EC changes. VEGF induced a rapid and prolonged reorganization of the F-actin cytoskeleton in ECs, manifested by the formation of stress fibers and focal adhesions. Y-27632, a specific inhibitor of Rho kinase, completely abolished the VEGF-induced cytoskeletal changes. Inhibition of Rho kinase had no effect on basal EC migration in response to mechanical wounding, but prevented the VEGF-enhanced EC migration. Furthermore, in an *in vitro* model for angiogenesis Y-27632 inhibited the VEGF-induced ingrowth of EC in a three-dimensional fibrin matrix in a dose-dependent manner. Thus, the VEGF-induced cytoskeletal changes in ECs are mediated by Rho kinase and activation of Rho kinase is involved in the VEGF-induced *in vitro* EC migration and angiogenesis.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from existing ones, is involved in a variety of diseases including tumor development, diabetic retinopathy and rheumatoid arthritis.[1-5] Four sequential steps can be distinguished in angiogenesis: an increased endothelial permeability associated with the degradation of the basal membrane and interstitial matrix, endothelial cell migration, endothelial cell (EC) proliferation and the formation of tubular structures with a lumen and a new basement membrane.[6] Angiogenesis is induced by angiogenic factors like Vascular Endothelial Growth Factor (VEGF), initially identified as a vascular permeability factor[7], and basic Fibroblast Growth Factor (bFGF). VEGF induces endothelial permeability, migration, proliferation, and angiogenesis[8] by multiple mechanisms including alteration of the F-actin cytoskeleton[9; 10] and induction of gene expression.[4; 5]

Reorganization of the F-actin cytoskeleton and cell-matrix adhesion play a crucial role in endothelial cell migration in angiogenesis and the repair of injuries along the endothelium. For cells to migrate, they must form new lamellipodia and adhere to the substratum at the front of the cell and detach from the substratum at the tail of the cell and retract their tail.[11] Formation of adhesive structures and cellular contraction are essential in this process. ECs contain cytoskeletal cables or bundles of actin and nonmuscle myosin filaments, that can contract and exert tension.[12; 13] A prominent group of these F-actin cables are the stress fibers (SFs), which are linked to the cell membrane at focal adhesions (FAs). VEGF is known to induce the formation of SFs and FAs *in vitro*.[9; 10; 14; 15] *In vivo* SFs occur mainly in large arteries[16; 17], to a lesser extent in the entire microcirculation[18; 19], but are largely absent from the venous system.[17; 20; 21] Many studies have shown that SFs develop during endothelial cell adaptation to unfavorable, or pathological situations including wound healing, atherosclerosis and hypertension.[16; 20; 22-24]

It has become well established that the the formation of SFs and FAs is induced by Ras-related GTPases of the Rho-family. Indeed it was found that Rho-like small GTPases regulate cell motility [25-27] in a variety of cell types. Using C3 transferase, a specific inhibitor of Rho proteins, Aepfelbacher *et al.* showed that Rho is involved in wound-induced formation of SFs and endothelial migration in an *in vitro* wound healing assay.[28] C3 transferase did not affect wound-induced formation of lamellipodia and filopodia, which are dependent on the activation of the Rho-related small GTPases Rac and Cdc42 respectively.

RhoA is coupled to $G\alpha_{13}$ via p115RhoGEF, a guanine nucleotide exchange factor, which activates RhoA.[29-31] Mice lacking $G\alpha_{13}$ failed to develop into an organized vascular system at E8.5, even though endothelial cells had been differentiated in these embryos.[32] These results indicate that $G\alpha_{13}$ is required for angiogenesis. Angiogenesis is dependent on migration, which may be defective in these mice since fibroblasts generated from these embryos do not migrate in response to $G\alpha_{13}$-coupled receptor agonists such as thrombin.[32] These results suggest that Rho-signaling might play a role in angiogenesis. Recently, Hla and coworkers found some evidence for the role of RhoA in sphingosine-1-phosphate-induced *in vitro* angiogenesis, which was completely blocked by C3 transferase, a specific inhibitor of RhoA.[33]

Numerous effectors have been identified for RhoA. The best characterized effector is the coiled coil forming Rho kinase[34-38], which is a likely target for RhoA, that could mediate the RhoA-induced cytoskeletal changes. Rho kinase was shown to be involved in the formation of SFs and FA complexes[39; 40] and to increase MLC phosphorylation.[35; 41] Uehata *et al.*[42] recently reported a synthetic pyridine-analog that inhibits the Rho kinase with high specifity compared to MLC kinase. This cell-permeant inhibitor, Y-27632, was able to prevent RhoA-mediated SF formation in smooth muscle cells.

Using Y-27632 we investigated in this study whether Rho kinase is involved in VEGF-induced *in vitro* endothelial cell migration and angiogenesis and whether Rho kinase might play a role in the accompanying cytoskeletal changes.

MATERIALS AND METHODS

Materials

Medium 199 supplemented with 20 mmol/L HEPES, L-glutamine and penicillin/streptomycin were obtained from Biowhittaker (Verviers, Belgium); newborn calf serum (NBCS) was obtained from Gibco (Grand Island, NY). Tissue culture plastics were from Costar (Cambridge, MA). A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine hypothalamus as described by Maciag *et al.*[43] Human serum was obtained from a local blood bank and was prepared from 10 to 20 healthy donors, pooled, and stored at 4°C. Trypsin was purchased from Gibco, heparin and thrombin from Leo Pharmaceutical Products (Weesp, The Netherlands), and human fibrinogen from Chromogenix (Mölndal, Sweden). Factor XIII was generously provided by Dr.H. Boeder and Dr.P. Kappus (Centeon Pharma, Marburg, Germany). bFGF was purchased from Pepro Tech EC (London, UK), and human recombinant TNF-α was a gift from Dr.J. Travernier (Biogent, Gent, Belgium) and contained 2.35×10^7 U/mg protein and less than 40 ng of lipopolysaccharide per mg of protein. VEGF was a gift from Dr.H.A. Weich (Braunschweig, Germany). Rhodamine phalloidin was from Molecular Probes (Eugene, OR, USA). Anti-vinculin Ig was obtained from Sigma Chemical Company (St. Louis, MO). Rabbit anti-mouse IgG-FITC from Dakopatts (Denmark). Y-27632 was supplied by Yoshitomi Pharmaceutical Industries (Saitama, Japan).

Cell Culture

Human foreskin microvascular endothelial cells (hMVECs) and human umbilical vein endothelial cells (HUVECs) were isolated, cultured, and characterized as previously described.[44-46] ECs were cultured on fibronectin- or gelatin-coated dishes in Medium 199 supplemented with 20 mmol/L HEPES (pH 7.3), 10% human serum, 10% heat-inactivated NBCS, 150 mg/ml crude ECGF, 2 mmol/L L-glutamine, 5 U/ml heparin, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37°C under 5% CO_2 /95% air atmosphere. Experiments with hMVECs and HUVECs were performed after they reached confluency and hMVECs were used after culture without growth factor for at least 24 hours.

Migration assay

After reaching confluence, hMVECs were cultured for 3 to 5 more days until a homogenous, quiescent monolayer with the typical cobblestone morphology developed. Wounds were made with a sterile pipette tip. Before wounding EC monolayers were pretreated for 30 minutes with 10 µmol/L Y-27632 and Y-27632 remained present for the next 24 hours. Immediately after wounding and at the end of the experiment (= after 24 hours), wounds were photographed and semiquantitative measurements were made of control and treated wounds. A mean wound width was determined and an average percentage wound closure was calculated.

In Vitro Angiogenesis Model

In vitro angiogenesis assays were performed as descibed previously.[47-49] Human fibrin matrices were prepared by the addition of 0.1 U/mL thrombin to a mixture of 5 U/mL factor XIII (final concentrations), 2 mg/mL fibrinogen, 2 mg/mL sodium citrate, 0.8 mg/mL NaCl, and 3 mg/mL plasminogen in indicator-free Medium 199, and 300-mL aliquots of this mixture were added to the wells of 48-well plates. After clotting at room temperature, the fibrin matrices were soaked with indicator-free M199 supplemented with 10% (v/v) human serum and 10% (v/v) NBCS for 2 hours at 37°C to inactivate the thrombin. Highly confluent hMVECs (0.7 x 10^5 cells/cm^2) were detached and seeded in a 1.25:1 split ratio on the fibrin matrices and cultured for 24 hours in indicator-free Medium 199 supplemented with 10% human serum, 10% NBCS, and penicillin/ streptomycin. The endothelial cells were then stimulated with the mediators for the time indicated. Every second day, the culture medium was renewed. Invading cells and the formation of tubular structures of endothelial cells in the three-dimensional fibrin matrix were analyzed by phase contrast microscopy, and the mean length of tube-like structures of six randomly chosen microscopic fields (7.3 mm^2/field) was measured using an Olympus CK2 microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software and expressed as a percentage of control.

Immunocytochemistry

The presence of vinculin and F-actin were visualized by indirect immunofluorescence with mouse anti-vinculin antibody (1:300) and by direct staining with rhodamine-phalloidin (1:100) in ECs grown on glass coverslips, as described previously.[50]

Statistical analysis

Data are reported as mean ± SD. Comparisons between more than two groups were made by one-way analysis of variance (ANOVA), followed by Bonferoni-adjusted chi-square test. Differences were considered significant at the $P < 0.05$ level.

RESULTS

VEGF-induced changes in the EC cytoskeleton require Rho kinase activity

To study the effects of VEGF on the endothelial cytoskeleton, ECs were grown on glass cover slips and stained for F-actin with rhodamine-phalloidin. Non-stimulated EC monolayers appeared as tightly connected cells, in which the cells displayed a characteristic peripheral rim of F-actin. Occasionally a few cytoplasmic F-actin filaments were observed (Figure 1A). Stimulation of the cells with 10 ng/mL VEGF resulted in an increase in cytoplasmic F-actin staining after 10 and 30 minutes (Figure 1B, and C), accompanied by the loss of the peripheral F-actin band. After 1 hour many SFs were present (Figure 1D), that remained visible after 2 hours (Figure 1E) and were less pronounced after 3 hours (Figure 1F). VEGF also induced the formation of small gaps between neighbouring cells (indicated with an arrow in Figure 1D).

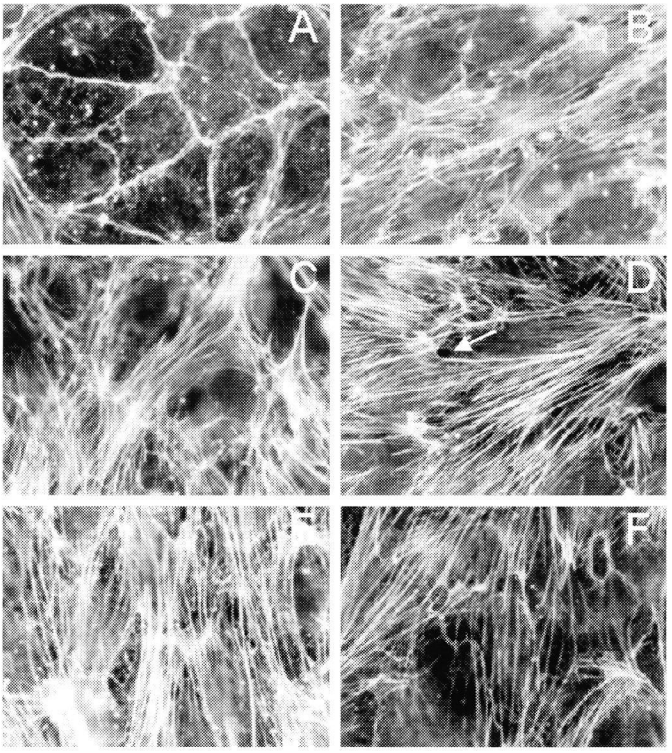

Figure 1. VEGF induces the formation of F-actin stress fibers in ECs.

Staining of F-actin in HUVEC. ECs were preincubated for 1 hour in Medium 199 + 1% HSA and stimulated with 10 ng/mL VEGF for 10 min (B), 30 min (C), 60 min (D), 120 min (E), 180 min (F), or non-treated (A). Arrow in D indicates the presence of a small gap between neighbouring ECs. The cells were stained as described in Materials and Methods. Similar results were obtained in three independent experiments. Bar, 10 μm.

To investigate whether Rho kinase was involved in the VEGF-induced SF formation, EC monolayers were preincubated with 10 μmol/L Y27632 for 1 hour. Y-27632-pretreated cells got a wrinkled appearance, but the cells remained tightly connected (compare Figure

2B with 2A). Preincubation with Y-27632 completely abolished the VEGF-induced formation of SFs (compare Figure 2D with 2C).

The VEGF-induced SF formation was accompanied by the formation of FAs as was evidenced by immunostaining for vinculin, a marker for the presence of FAs. Under basal conditions vinculin appeared as a thin discontinous peripheral rim in ECs (Figure 2E), which was not altered by preincubation with Y-27632 (Figure 2F). After stimulation with VEGF vinculin was organized in discrete spots, indicating the formation of FAs (Figure 2G). Inhibition of Rho kinase prevented the formation of FAs (Figure 2H). Thus, the VEGF-induced changes in the endothelial require activation of Rho kinase.

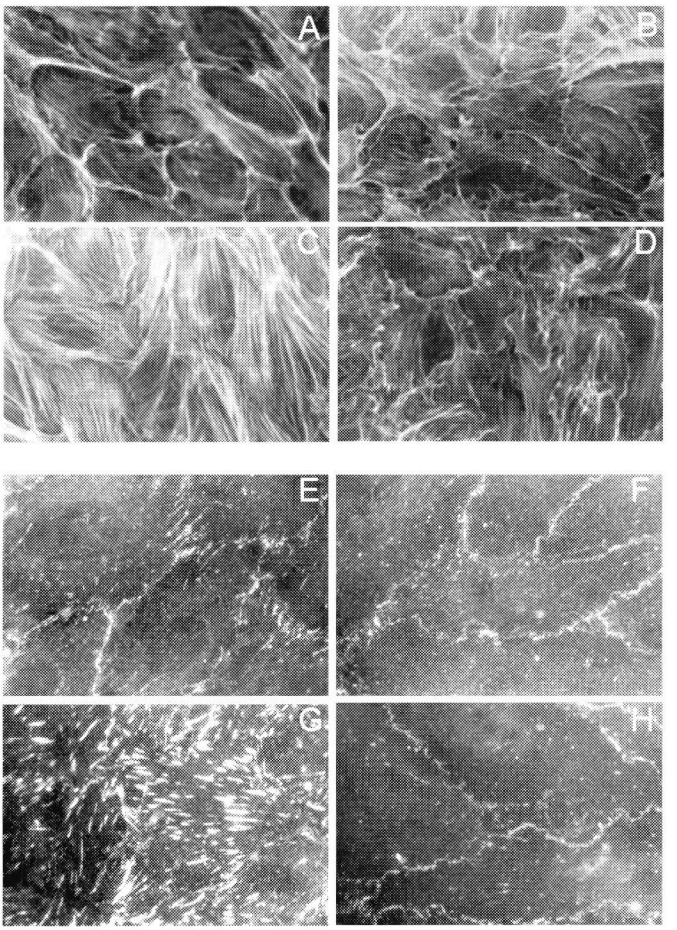

Figure 2. Rho kinase is involved in the VEGF-induced formation of F-actin stress fibers and focal adhesions in ECs. Immunocytochemical staining for F-actin (A through D) and vinculin (E through H). HUVECs were preincubated for 1 hour in Medium 199 + 1% HSA in the presence or absence of 10 μmol/L Y-27632 and subsequently stimulated with 10 ng/mL VEGF or vehicle-treated. The cells were stained as described in Materials and Methods. A and E Basal condition. B, D, F, and H, Cells were preincubated with Y-27632. C, D, G, and H, Cells were stimulated for 60 minutes with VEGF. Similar results were obtained in three independent experiments and in hMVEC monolayers. Bar, 10 μm.

Inhibition of Rho kinase reduces VEGF-induced, but not basal cell migration

Stimulation of ECs with VEGF was shown previously to enhance endothelial migration in an *in vitro* model of EC responses to mechanical wounding.[51] To investigate whether Rho kinase is involved in the VEGF-enhanced endothelial migration, confluent and quiescent monolayers of hMVECs cultured on fibronectin-coated dishes were wounded by removing cells with a pipette tip and wound closure was measured after 24 hours. Recovery of these monolayers is dependent on migration only, because proliferation of ECs in response to wounding does not start before 24 hours.[51] Under our conditions, cells migrated into the cell-free area from both wound edges until wound closure was reached within 48 hours (data not shown). 24 hours after wounding the percentage of wound closure under nonstimulated conditions was 52 ± 9, so that both stimulation and inhibition of EC migration could be determined (Figure 3B).

Treatment with 10 ng/mL VEGF enhanced endothelial migration significantly (Figure 3B). In the presence of 10 µmol/L Y-27632 basal EC migration was slightly but not statistically significantly inhibited when compared to control conditions. However, coincubation of VEGF with Y-27632 abolished the VEGF-enhanced migration completely (Figure 3B). This data indicates that inhibition of Rho kinase does not alter basal EC migration, but prevents the VEGF-induced EC migration.

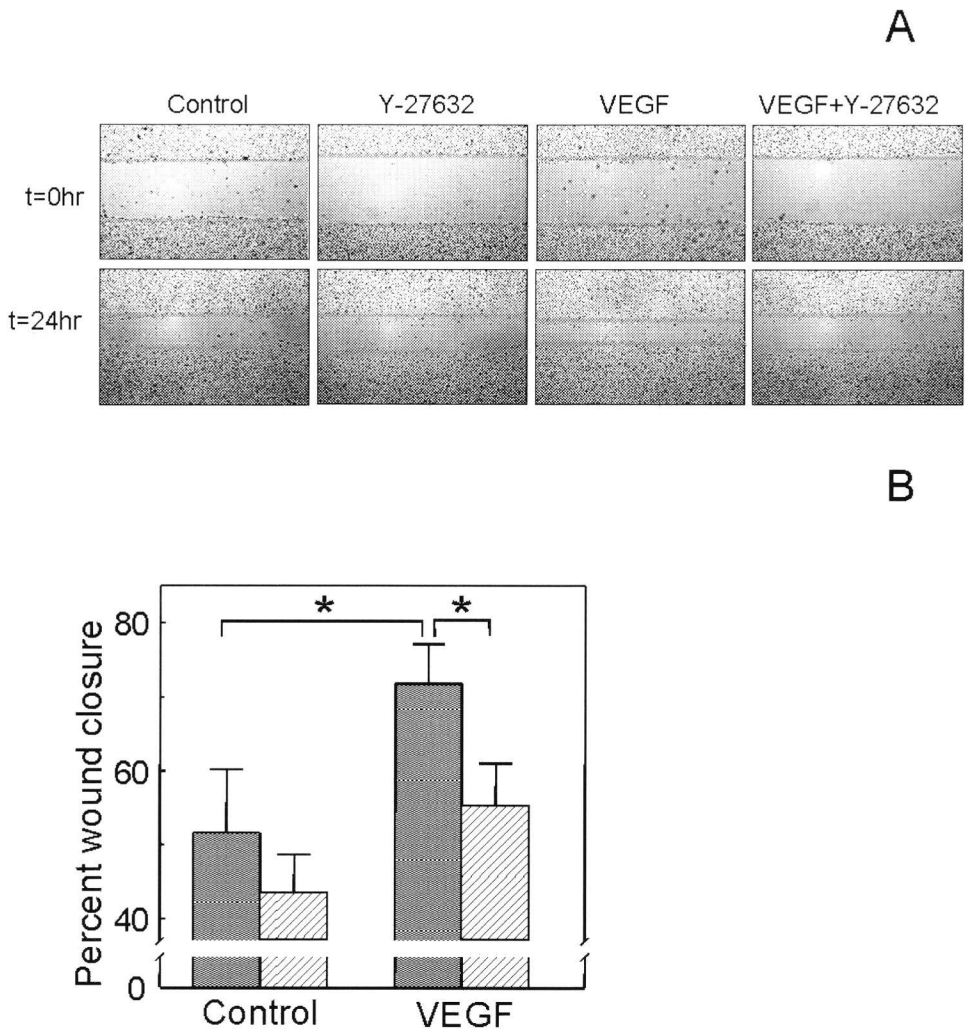

Figure 3. Effects of Y-27632 on endothelial cell migration *in vitro*.

A: Confluent monolayers of hMVECs cultured on fibronectin were pretreated with 10 µmol/L Y-27632 for 1 hour when indicated, and wounded with a pipette tip. Subsequently the ECs were stimulated with 10 ng/mL VEGF or vehicle-treated. Photographs were taken directly after wounding (t = 0 hr) and 24 hours after wounding (t = 24 hr). Bar, 300 µm.

B: Quantification of the endothelial wound repair. ECs were pretreated with 10 µmol/LY-27632 for 1 hour (hatched bars) or sham-treated (filled bars), wounded, and stimulated for 24 hours with VEGF when indicated. After 24 hours endothelial cell migration was quantified. Values are the mean ± SD from 2 experiments in triplicate. *P<0.05

Role of Rho kinase in the formation of tubular structures by hMVECs in a fibrin matrix

Stimulation with a combination of VEGF and TNF-α (VEGF/TNF-α) induced the formation of tubular structures invading the fibrin matrix when hMVECs were cultured on top of the fibrin matrix (Figure 4B and ref[47]). The confluent monolayer remained unaltered in the absence of growth factors or cytokines (Figure 4A).

Y-27632 was used to establish the involvement of Rho kinase in the formation of capillary-like tubular structures by hMVECs in a three-dimensional matrix. Simultaneous incubation of the VEGF/TNF-α-stimulated monolayers for 7 days with Y-27632 did not alter the morphology of the EC monolayers on top of the fibrin matrix or attachment of the cells to the fibrin matrix, indicating that Y-27632 had no toxic effects on the ECs (Figure 4C and 4D).

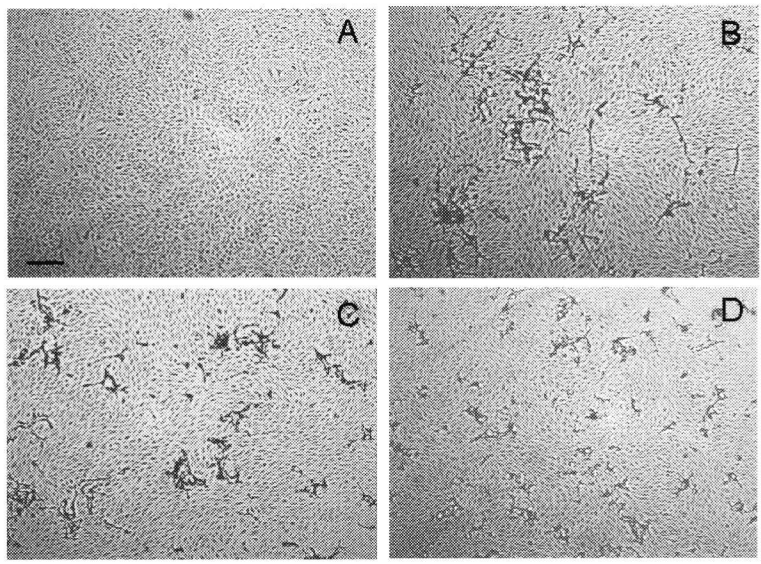

Figure 4. Capillary-like tube formation is reduced by inhibition of Rho kinase.
hMVECs were cultured on top of a three-dimensional fibrin matrix in Medium 199 supplemented with 10 % human serum and 10% NBCS and were not stimulated (A) or stimulated with a combination of 25 ng/mL VEGF and 1 ng/mL TNF-α (B, C and D) either in the absence (B) or in the presence of 1 μmol/L Y-27632 (C) or 10 μmol/L Y-27632 (D). After 7 days of culture, non-phase photomicrographs were taken. Bar, 300 μm.

Treatment with Y-27632 reduced the mean tube length of the capillary-like tubular structures formed in response to VEGF/TNF-α in a dose-dependent manner (Figure 5A). However, at a maximally inhibiting concentration of 10 μmol/L Y-27632, the number of the

onsets of tube-like structures that started to form in response to VEGF/TNF-α increased (Figure 4D and 5A).

Similar results were obtained when VEGF was replaced by bFGF to stimulate the *in vitro* angiogenesis (Figure 5B).

Thus, these data indicate the dual role of Rho kinase in the formation of tubular structures by hMVECs stimulated by either VEGF or bFGF. Rho kinase activity is necessary for the proper ingrowth of ECs in the three-dimensional matrix, and restricts the number of ECs that start to develop tubular structures.

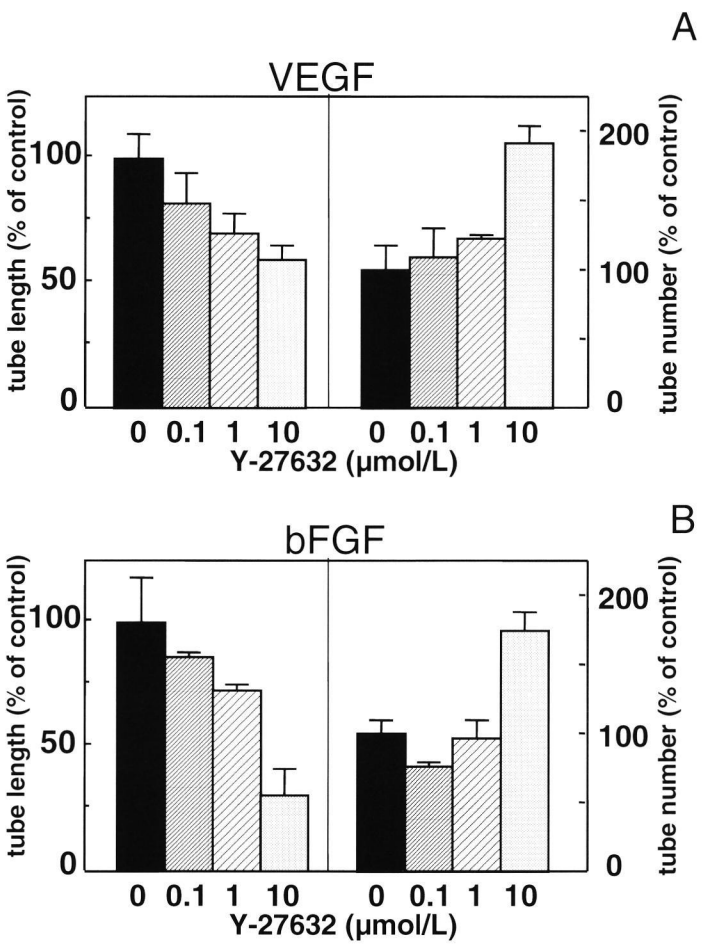

Figure 5. Dose-dependent reduction of the mean tube-length of growth factor-induced capillary-like tubes by inhibition of Rho kinase with Y-27632, which is accompanied by an induction of the number of tube-like structures formed.

A: Effect of Y-27632 on the mean tube length (left panel) and mean tube number (right panel) formed by VEGF/TNFα-stimulated hMVEC monolayers. After 7 days of culturing the number of tube-like structures/cm^2 was determined and expressed as a percentage of the mean number of tubes/cm^2 in the absence of Y-27632 (right panel). Total tube length/cm^2 was measured and divided by the number of tube-like structures/cm^2 to obtain the mean tube length. Mean tube length was expressed as a percentage of the mean tube length in the absence of Y-27632 (left panel). Values are the mean ± SD of 2 independent experiments in triplicate.
B: Effect of Y-27632 on the mean tube length (left panel) and mean tube number (right panel) of the tube-like structures formed by hMVEC monolayers, stimulated with a combination of bFGF (5 ng/mL) and TNFα (1 ng/mL). Values are the mean ± SD of 2 independent experiments in triplicate.

DISCUSSION

The major finding of this study is that inhibition of Rho kinase reduced growth factor-induced endothelial migration and angiogenesis *in vitro*, and abolished the accompanying growth factor-induced changes in the endothelial cytoskeleton.

Stimulation of endothelial monolayers with VEGF induced the formation of SFs and FAs, in accordance with other reports.[9; 10; 14; 15] These SFs were formed rapidly and maintained for at least 3 hours. They probably exist must longer. Cohen *et al.* reported the expression of SFs in ECs 45 hours after VEGF treatment.[9] Induction of SFs and FAs involves Rho kinase. Transfection of constitutive active Rho kinase in Swiss 3T3 cells results in the formation of SFs and FAs, although they were slightly different from agonist-induced SFs and FAs. Inhibition of Rho kinase activity either by Y-27632 or by dominant negative Rho kinase reduces the cytoskeletal effects of activators of RhoA as LPA and thrombin, and even of shear stress.[42; 52] In the present study we show for the first time that Rho kinase is also involved in the VEGF-induced cytoskeletal changes.

Migration is crucial for the repair of injured blood vessels, angiogenesis, and atherogenesis and is accompanied by the formation of SFs *in vivo*.[22; 53] Conflicting results have been reported with regard to the role of RhoA and Rho kinase in cell migration. Aepfelbacher *et al.* found that inhibition of RhoA with a high concentration of C3 transferase attenuated HUVEC migration.[28] Nobes and Hall showed that treatment of fibroblast monolayers with C3-transferase had no effect on wound closure, and Y-27632 even slightly enhanced wound closure.[54] Here we show that these apparent contradictory data probably result from differences in the type of stimulus for migration. In our hands Y-27632 did not affect basal cell migration significantly in accordance with the data of Nobes and Hall. However, Y-27632 did inhibit VEGF-induced endothelial migration. This fits with the data

from Aepfelbacher *et al.*, as they performed their experiments in the presence of an endothelial growth supplement.

Angiogenesis requires extensive adaptations of the shape and cytoskeleton of ECs. It is remarkable that no evidence currently is available about the cytoskeletal changes that accompany angiogenesis *in vivo*. Our finding that Y-27632 inhibited the ingrowth of endothelial cell in a 3-dimensional matrix, indicates that Rho kinase activity is necessary for angiogenesis.

Inhibition of Rho kinase did not prevent the onset of the *in vitro* angiogenesis process. At a maximal effective concentration of 10 µmol/L, treatment with Y27632 even increased the number of capillary-like tubes. This means that the angiogenic switch is not effected by Y-27632, but that the ingrowth in the fibrin matrix is inhibited. This is likely to be the result of the disturbed migratory capability of the ECs. The increase in tube number probably reflects a lower adhesive capability that allows endothelial remodeling, as treatment with Y-27632 resulted in the loss of FAs, the anchoring structures of the cell cytoskeleton to the matrix.

A 7-day treatment of confluent endothelial monolayers with 10 µmol/L Y-27632, a concentration which was previously shown to inhibit Rho kinase activity completely, did not change cell morphology and had no toxic effects. This demonstrates that Rho kinase activity is not necessary at all under resting conditions, but is only involved in cell shape change or remodeling processes. This explains why *in vivo* Y-27632-treatment had no effect on normal blood pressure, whereas it reduced elevated blood pressure in several rat hypertension models.[42]

It is not likely that Rho is the only small GTPase involved in angiogenesis, as recently p21-activated kinase (PAK), an effector of the Rho-like GTPases Rac and Cdc42 but not Rho, was shown to be involved in (endothelial) migration.[11; 55] In other cell types Rac and Cdc42 are known to be involved in cell migration[27; 54], but to the best of our knowledge no direct evidence for the involvement of Rac and Cdc42 or their effectors in angiogenesis exists.

The process of angiogenesis *in vivo* is preceded by an increase in endothelial permeability. This increase in permeability results in the formation of a fibrinous exudate and the laying down of a provisional matrix, providing an excellent situation for the ingrowth of endothelial cells. We and others have previously shown that an increase in endothelial permeability as can be induced by thrombin, lyso-phosphatidic acid and mildly oxidized LDL particles is mediated by activation of Rho and Rho kinase.[50; 56; 57] Here we show that the VEGF-induced changes in the EC cytoskeleton are accompanied by the formation of small gaps between neighbouring ECs, indicative of endothelial barrier dysfunction. Recently,

evidence was provided that Y-27632 inhibits the VEGF-induced endothelial permeability.[58] These data support the idea that the changes that occur in microvascular endothelial cells which contribute to increased permeability, also contribute to the generation of a pro-angiogenic state of the endothelium.

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8

GENERAL DISCUSSION

GENERAL DISCUSSION

The starting point for the present study was the gap of knowledge regarding the mechanisms involved in prolonged types of vascular leakage. The initial phase of endothelial barrier dysfunction in inflammation is well characterized and can be mimicked by activation of healthy microvessels with inflammatory mediators like histamine, bradykinin and Platelet Activating Factor. It is known to be Ca^{2+}/Calmodulin- and MLC kinase-dependent. This type of endothelial barrier dysfunction is very transient and comparable to the mosquito bite-type of vascular leakage. Under normal conditions this transient endothelial barrier dysfunction is not life-threatening. However, often severe and prolonged vascular leakage occurs, which is not treatable with antihistaminergic agents. Regarding the mechanism(s) of the sustained endothelial hyperpermeability less is known. For a suitable treatment of prolonged hyperpermeability it is necessary to obtain more insight into the underlying processes which cause a prolonged endothelial barrier dysfunction, as current therapies failed to reduce prolonged vascular leakage. The major finding of the studies described in this thesis is that RhoA/Rho kinase signaling under distinct conditions contributes to the prolongation of endothelial barrier dysfunction at least *in vitro*. Furthermore, evidence was provided that the widely-used cholesterol-lowering drug simvastatin reduced prolonged endothelial hyperpermeability in an *in vitro* model and improved endothelial barrier function in an *in vivo* rabbit model.

Signal transduction pathways involved in the regulation of endothelial barrier function

It has been debated whether the *in vitro* model for endothelial permeability is comparable to the *in vivo* situation because previous studies with histamine showed a prolonged increase in permeability, while it is rapid and transient in the healthy microvasculature. In chapter 3 we distinguised for the first time in an *in vitro* model of human umbilical vein endothelial cells (HUVECs) grown on porous filters between transient and prolonged effects of vasoactive compounds on endothelial permeability. In a system without serum histamine induced a transient endothelial barrier dysfunction, while under identical conditions thrombin had a prolonged effect. This indicates that it concerns 2 different responses to induce endothelial hyperpermeability (see also table 1). Evidence was provided that a transient elevation of the $[Ca^{2+}]_i$ raised by histamine, and typical of many more vasoactive agents, induces a transient decrease in endothelial barrier function via an MLC kinase-dependent contractile process. Thrombin on the other hand induced a more sustained endothelial barrier dysfunction, which in addition to elevating the $[Ca^{2+}]_i$ was mediated by

activation of the small GTPase Rho. This was evidenced by inhibition of the Rho protein by the toxin C3 transferase, a specific inhibitor of Rho. C3 transferase reduced both the thrombin-enhanced MLC phosphorylation and endothelial barrier dysfunction, but had no effect on the histamine-induced permeability.

Table 1. Increase in endothelial permeability

*Histamine-type response

*Thrombin-type response

*Leukocyte-enhanced response

*Hydrostatic pressure

*Angiogenesis-associated response

*VEGF-type response

At the same time Essler *et al.* reported comparable data using C3 transferase and a dominant negative mutant of RhoA in HUVECs, supporting a role for Rho in the thrombin-enhanced endothelial permeability.[1] We hypothesized, that as in smooth muscle cell contraction, Rho could act in prolonged endothelial contraction via Ca^{2+}-sensitization. In bovine pulmonary artery EC monolayers inhibition of Rho with C3 transferase improves barrier function similarly to HUVEC.[2] C3 transferase, however, had no effect on thrombin-enhanced permeability in these bovine EC monolayers.[2] This indicates that species differences could exist regarding the involvement of signal transduction pathways in thrombin-enhanced hyperpermeability. Previously, studies to the involvement of protein kinase C in the regulation of endothelial barrier function identified the existence of species differences (see chapter 1 and 3 of this thesis).[3-7] So, one has to be carefull with the extrapolation of data about the regulation of barrier function obtained from animal studies to the human situation. Two reports from the same group reported that activation of Rho did not increase endothelial permeability of HUVEC monolayers.[8; 9] However, the basal permeability of the endothelial monolayers used in those studies was already high. Under such conditions a further disruption of endothelial barrier function is almost impossible and thus not measurable (even when thrombin was used as an permeability-increasing agent). To our knowledge no data are currently available regarding Rho activation in microvascular ECs and the role of Rho in the regulation of endothelial hyperpermeability *in vivo*.

In chapter 4 we showed that, in the absence of a rise in $[Ca^{2+}]_i$, activation of Rho by *lyso*-phosphatidic acid (LPA) was sufficient to induce an hyperpermeability response. LPA is a well-known Rho-activator. This demonstrates that Rho does not act only via sensitization of

the Ca^{2+}-induced hyperpermeability response, but itself is able to induce endothelial hyperpermeability independently of a rise in $[Ca^{2+}]_i$. This implies that Ca^{2+}-dependent and Rho-dependent signaling represent two pathways, which both can be activated independently and that activation of either one of these pathways results in a disruption of the endothelial integrity. In chapter 5 evidence was provided, that thrombin also is able to induce an increase in endothelial permeability via the activation of Rho, independently of a rise in $[Ca^{2+}]_i$. It was demonstrated that in endothelial monolayers in which the rise in $[Ca^{2+}]_i$ was prevented by the chelation of intracellular Ca^{2+}-ions thrombin still induced a Rho-dependent increase in endothelial permeability. Ca^{2+}-independent Rho-mediated barrier disruption now turns out to be a more general mechanism of increased endothelial permeability, as also bacterial toxin-induced endothelial permeability was demonstrated to act via this mechanism.[10]

In chapter 3 we provided evidence that genistein- and herbimycin A-sensitive protein tyrosine kinases (PTK) are involved in the prolonged thrombin-enhanced endothelial hyperpermeability, but not in transient endothelial barrier dysfunction. Other reports confirmed the involvement of PTK in thrombin-enhanced permeability.[11; 12] Evidence is now accumulating that PTK are involved in barrier dysfunction induced by a variety of vasoactive agents, including VEGF[12; 13], hydrogen peroxide[14], histamine[15], and LPS[16]. This underscores the general importance of PTK in the regulation of endothelial barrier function. All the indicated studies were performed *in vitro*. *In vivo* studies using protein tyrosine phosphatase inhibitors also demonstrated that protein tyrosine phosphorylation induced vascular leakage.[17]

In chapter 3 it was shown that PTK are involved in an increase in endothelial permeability under conditions in which the rise in $[Ca^{2+}]_i$ was prevented. In chapter 5 it was shown that PTK are involved in an increase in endothelial permeability under conditions in which activation of Rho kinase was prevented. Taken together this implies that activation of PTK is involved in endothelial barrier dysfunction independently of Ca^{2+}- and Rho-mediated signal transduction pathways and represents a third pathway resulting in an endothelial barrier dysfunction (see also figure 6 of chapter 5). *In vivo* experiments also suggested that it concerns an independent mechanism as the inhibition alone of protein tyrosine phosphatase activity in pig coronary venules increased endothelial permeability.[17] This does not exclude the fact that PTK are also involved in the other two pathways. On the contrary, evidence exists that PTK can play a role in both Ca^{2+}- and Rho-dependent endothelial hyperpermeability.

Many target proteins were identified that can be phosphorylated by PTK in ECs. Several of the PTK target molecules have been implicated in the regulation of the endothelial barrier function including MLC kinase [18], pp125FAK and paxillin[17], VE-cadherin and its associated catenins.[15] Tyrosine phosphorylation of the endothelial MLC kinase promotes its kinase activity and thus promotes EC contraction. This is in accordance with our finding that inhibition of PTK reduces MLC phosphorylation and barrier dysfunction. The experiments described in chapter 3 indicated however that PTK are also involved in additional Ca^{2+}-independent signaling pathways. So, it is likely that activation of MLC kinase by PTK does not represent the 'third' PTK-dependent pathway. Pp125FAK plays a role in the formation of focal adhesions. We showed that the formation of focal adhesions that accompanies the thrombin-enhanced endothelial permeability was prevented by the inhibition of PTK. Formation of focal adhesions is known to be RhoA- and Rho kinase-dependent.[19] Inhibition of Rho with C3-transferase (chapter 4) or Rho kinase with Y-27632 (chapter 7) indeed prevented the formation of focal adhesions. However, inhibition of PTK further reduced endothelial barrier dysfunction under conditions where RhoA/Rho kinase signaling was prevented. This indicates that it is likely that phosphorylation of pp125FAK by PTK also does not represent the 'third' pathway.

PTK-dependent dissociation of the VE-cadherin/catenin complexes might represent this third pathway. These adherens junctional proteins are responsible for the tethering forces between ECs.[20; 21] Abolition of tethering results in EC contraction as a consequence of the presence of a basal isometric tension. Thrombin-induced dissociation of $\beta\gamma$-catenins from the cytoskeleton was not prevented by C3 transferase and indicates that thrombin-induced dissociation of the VE-cadherin/catenin complex is Rho-independent.[1]

Pathophysiological implications

An interesting finding of our studies was that a prolongation of the histamine-induced endothelial barrier dysfunction was observed after the addition of serum to the experimental system. Serum contains among other things a high concentration of LPA. In chapter 4 we provided evidence that stimulation of endothelial cells with LPA indeed induces a sustained endothelial contraction. So, Rho activation by LPA could be responsible for the prolongation/sensitization of the histamine-induced reaction, but this remains to be proven experimentally. It is likely that a similar sensitization mechanism contributes to the leukocyte-enhanced endothelial hyperpermeability (see below), and possibly also to the prolonged edema induced by circulating humoral factors. Testing the effects of sera of patients suffering

from prolonged edema on the histamine-induced permeability in our *in vitro* system should answer this question, and allows one to identify such circulating factors.

It remains to be investigated which inflammatory mediators induce activation of Rho in ECs. A plethora of mediators is released during the inflammatory response. TNF-α, produced by macrophages and monocytes, is one of the possible candidates. Some studies indicate that TNF-α induces endothelial barrier dysfunction via gap formation between endothelial cells[22], and the effects of TNF-α on the endothelial cytoskeleton and gap formation are Rho-dependent.[23] Other studies, however, reported the absence of a direct effect of TNF-α on the permeability of endothelial monolayers[24; 25] or reported an increase in pulmonary edema that was entirely caused by increased neutrophil sequestration.[26] An inflammatory mediator-independent scenario of Rho-mediated endothelial hyperpermeability is also possible.

Evidence is now accumulating that adhesion of circulatory cells to the endothelium directly activates Rho signaling in the endothelium, without the involvement of an intermediate inflammatory mediator. Activation of Rho in endothelial cells in this way might facilitate the transmigration of these cells across the endothelium, by creating small pores in the endothelial barrier comparable to those involved in the passage of macromolecules (see this thesis). Monocyte adhesion and spreading on HUVEC is dependent on Rho, but not on Rac and Cdc42 activities in endothelial cells.[27] Furthermore, adherent neutrophils induce the formation of stress fibers in endothelial cells.[28-30] The major evidence for the involvement of Rho in leukocyte extravasation is provided by studies on lymphocyte transmigration across blood brain barrier endothelial cells.[31; 32] In a review Butcher and Picker[33] proposed a multistep model of lymphocyte-endothelial cell recognition and recruitment of lymphocytes from the blood: (1) Contact through microvillous receptors/rolling of lymphocytes (2) Activation of lymphocytes through G protein-linked receptors which trigger (3) integrin adhesion to vascular ligands in seconds through an intracellular pathway involving the small GTP-binding protein Rho followed by (4) diapedesis. With some minor modifications this model is probably applicable to transmigration of other circulatory cells. In this model lymphocyte integrin clustering and adhesion to their counterreceptors on the endothelium takes a central place. Integrin clustering in the endothelium also causes activation of Rho and the formation of stress fibers and focal adhesions. These events may contribute to the facilitation of the migration of circulatory cells. Besides in the transmigrating cell, integrin-mediated adhesion can also activate Rho signaling in the endothelial cell, and thus cause endothelial contraction.

Our finding that Rho signaling is involved in thrombin-enhanced endothelial permeability might have a broader implication than identification of a possible role for Rho in endothelial hyperpermeability in inflammation only. We observed that LPA also increased permeability. This identifies LPA as a new vasoactive substance possibly involved in vascular leakage. LPA is thought to be formed by activated platelets and was recently also shown to represent much of the endothelial cell-activating activity of oxidized LDL.[34] The finding that the effects of mildly oxidized LDL on endothelial permeability are dependent on activation of Rho kinase[35] is of particular interest in this context, as endothelial integrity is decreased during the development of atherosclerosis. Thus, this suggests that LPA may play an important role in the endothelial hyperpermeability of atherosclerotic vessels via the activation of Rho signaling.

As LPA is a release product of activated platelets it would be of interest to further investigate whether LPA could also be involved in other vascular disorders associated with an increased endothelial permeability.

Several bacterial toxins have been shown to activate Rho, including Cytotoxic necrotizing factor-1 (CNF)[9] and *Pasteurella multocida* toxin (PMT).[10] Of these toxins PMT was shown to induce HUVEC permeability via Rho/Rho kinase signaling.[10] Infections with *Pasteurella multocida* are characterized by acute inflammatory symptoms of the skin, such as edema and are often complicated by sepsis.[36] The toxin itself induces edema in animals.[37] Thus, Rho signaling also might contribute to the edema developed by bacterial infections.

Therapeutic implications

The studies presented in this thesis add two new potential ways to reduce endothelial hyperpermeability to the existing strategies (see table 2). As discussed in Chapter one of this thesis current therapies often fail to reduce prolonged vascular leakage: blockage of (histamine) receptors is only applicable in acute situations. Desensitization occurs after 1 or 2 days in the case of elevation of intracellular cAMP levels by adrenergic agents. Application of the principal of elevation of intracellular cGMP levels is limited because its benefits are restricted to particular parts of the vascular bed, whereas in other parts of the vascular bed the contrary will occur. To the best of our knowledge no clinical data are currently available with regard to the reduction of leukocyte-endothelium interaction.

Table 2. Reduction of endothelial permeability

*Receptor blockage

*General response: cAMP

(incl. histamine-, thrombin-, and leukocyte enhanced-type)

*Cell-type specific response: cGMP

(thrombin- and oxidant-increased permeability)

*Reduction of leukocyte-endothelium interaction

**Inhibition of RhoA/Rho kinase signaling*

**Statin treatment*

Our discovery of the role of RhoA/Rho kinase signaling in the disruption of the endothelial barrier function identifies Rho kinase as a new therapeutic target for possible clinical intervention. Inhibiting Rho kinase activity is preferable above inhibiting RhoA, as a higher specificity is acquired when inhibiting Rho kinase. RhoA is involved in many basal functions of the cell including cell division, and gene regulation and activates many downstream targets. Inhibition of just one of these targets, Rho kinase, turned out to be sufficient to prevent RhoA-mediated endothelial barrier disruption. Thus, inhibition of Rho kinase instead of RhoA minimizes the change of unwanted side-effects to occur. A striking feature of the inhibition of Rho kinase activity is, that it had no effect on basal endothelial permeability, neither was basal endothelial cell migration affected after wounding. From the *in vitro* angiogenesis experiments described in chapter 7 it appeared that the inhibition of Rho kinase activity over seven days did not disturb the endothelial monolayers and that endothelial cells remained viable under these conditions. This indicates that in resting endothelial monolayers there is a low degree of absence of Rho kinase activity. The same was observed when Rho kinase was inhibited *in vivo*.[38; 39] Inhibition of Rho kinase *in vivo* had no effect on heart rate, normal blood pressure, blood and urine chemistry, but Rho kinase was shown to be involved in pathological processes. Inhibition of Rho kinase reduced blood pressure in several rat hypertension models and vasospasm in a pig coronary spasm model. Rho kinase inhibitors can be used *in vivo* no toxic effects have so far been reported.[38-40] Thus, this suggests that Rho kinase activity is not required under basal conditions and is involved especially in pathological processes. However, caution should be observed as hypertension and vascular leakage often occompany each other. Even a local elevation of blood pressure can occur at sites of elevated extravasation of plasma proteins, that is not manifested by a general hypertension. Furthermore, these studies were performed in

animals, which were otherwise healthy. This is surely not the case in patients suffering from severe vascular leakage, as they are often in a bad general condition.

A second mechanism identified as playing a role in increased endothelial permeability is the activation of PTK. As outlined above PTK seem to be involved in a variety of conditions with increased endothelial permeability. Whether this finding is useful for clinical practice remains to be seen. Application of this knowledge is hampered by the fact that the identity of the PTK involved is largely absent, so that specific inhibition of one single PTK is still not possible.

The discovery that treatment with simvastatin attenuated the thrombin-enhanced endothelial permeability *in vitro* and improved endothelial integrity *in vivo* under atherosclerotic conditions could have greater clinical consequences for the treatment of vascular leakage. This finding was a direct result of our studies on the role of Rho in endothelial permeability. In the mean time statins were demonstrated in addition to their lipid-lowering effects to improve endothelial function and inhibit Rho function. Besides lowering plasma cholesterol levels an improvement of the endothelial integrity could contribute to the inhibitory effect of statins on the progression of the development of atherosclerosis. Although an decreased barrier function is firmly established as accompanying the development of atherosclerosis it is still not known whether this precedes the development of atherosclerosis or is a consequence of it. Statin therapy is also associated with reduced restenosis rate and improved clinical outcome following coronary stent implantation.[41] Improvement of endothelial barrier could contribute to these effects of statins, as stent implantation is accompanied by an increased endothelial permeability even when the endothelium is fully recovered as was evidenced by electron microscopy.[42] A major advantage of statins above Rho kinase inhibitors is the wealth of clinical experience in the use of statins as lipid-lowering drugs, which are currently regarded as safe.

Besides their application in atherosclerosis statins could find a new role as inhibitors of vascular leakage in inflammation. First of all more has to be learned about the effects of statins on the microvasculature, as vascular leakage in inflammation occurs in the postcapillary venules. The recent finding that simvastatin decreases leukocyte extravasation in rat mesenterium indicates that statins act on the microvasculature[43] and increases the chance of a positive outcome for statin treatment in the treatment of vascular leakage. Secondly, more has to be learned about the time course of the improvement of the endothelial barrier by statin treatment. In the *in vitro* experiments statin treament was already effective as early as after a 4-hour incubation period and the benefits of simvastatin remained present for at least 48 hours. No longer incubation periods were tested. This

means that the effects of statins on endothelial barrier function have a rapid onset. Improved endothelial integrity *in vivo* was observed after a four-weeks period of simvastatin treatment of Watanabe rabbits. With respect to lowering plasma cholesterol levels a 4-week treatment is very short and only resulted in an insignificant tendency to decrease plasma cholesterol levels. With respect to the benefits of simvastatin on endothelial integrity 4 weeks is a rather long period. Treatment of vascular leakage with β–adrenergic agents resulted in a desensitization 1 or days after the start of the treatment.[44; 45] This might imply that simvastatin could be a suitable alternative for the treatment of prolonged endothelial hyperpermeability. As statins are able to inhibit Rho function, it is also worthwhile investigating whether statin therapy could reduce vascular leakage induced by poisoning with bacterial toxins, which act via the activation of Rho.[10] Even endothelial hyperpermeability associated with angiogenesis and the development of tumors could be a target to test. Recent evidence indicates that statins prevent angiogenesis *in vitro*.[46] In chapter 7 we provided evidence that similar Rho kinase-dependent processes are involved in endothelial hyperpermeability and angiogenesis. To summarize, statin treatment seems to be a promising new strategy for the treatment of Rho-mediated vascular leakage, but requires further research.

Limitations of the study

The *in vitro* model used in the studies described in this thesis provides a well-established model for studying endothelial permeability. However, every *in vitro* model remains an approximation of the *in vivo* situation. The power of the model used is that the contribution of the endothelium to vascular barrier function can be specifically investigated under well-defined conditions. At the same time this raises several drawbacks of the model, as vascular leakage comprises more than the contribution of the endothelial barrier only. Other factors that contribute to vascular leakage include elevated blood pressure, involvement of leukocytes, and reduced drainage by lymphatic vessels.[47] These factors are absent in the *in vitro* model. In the *in vitro* model endothelial hyperpermeability was induced by activation of the endothelium by one single mediator. *In vivo* often more than one vasoactive compound is responsible for increased vascular permeability. This is especially clear under inflammatory conditions, where a plethora of barrier-disrupting agents is involved in barrier dysfunction. It is the sequential release of these factors, that is thought to be responsible for the prolongation of the vascular barrier dysfunction. Furthermore, one has to realize that regional differences in the regulation of endothelial barrier function exist, e.g. in

inflammation especially the postcapillary venules become leaky[47], and cGMP is known both to improve and decrease barrier function depending on the part of the vascular bed involved. As long as one realizes these limitations one can use the *in vitro* model to study the regulation of endothelial barrier function, but results obtained from *in vitro* studies always have to be compared with and confirmed in suitable *in vivo* models. Our finding that simvastatin treatment reduced endothelial barrrier dysfunction both *in vitro* and *in vivo* indicates that the *in vitro* model is a suitable tool for starting the investigation of endothelial barrier function.

Future research

Several unanswered questions have already been mentioned above, such as what is the role of LPA *in vivo*; which inflammatory mediators could act via Rho; is LPA involved in vascular disorders associated with enhanced endothelial permeability other than atherosclerosis; which PTKs are involved in the regulation of endothelial barrier function, and under which conditions could statin treatment aid the reduction of vascular permeability? Some other unsolved themes, however, are of particular interest and may contribute to a better understanding of the regulation of the endothelial barrier function. They will be discussed here.

First of all, the role of Rho proteins in endothelial permeability/vascular leakage *in vivo* has to be established, as no reports have appeared thus far concerning the role of Rho proteins in endothelial permeability *in vivo*. From the studies described in this thesis it is likely that Rhoplays a role in several distinct types of endothelial hyperpermeability *in vivo*. An initial approach to studying the role of Rho *in vivo* is the investigation of the Rho function in hyperpermeability of isolated microvessels induced by inflammatory mediators. Subsequently, it is necessary to investigate the involvement of Rho in the regulation of vascular barrier function in intact animals. The recent development of administration of the Rho inhibitor C3 transferase by the use of an osmotic minipump raises new possibilities for performing this.[48] A major advantage of the mouse model is the availability of transgenic mice with specific properties. This allows the investigation of the role of the endothelial barrier in specific disease models. New Rho kinase inhibitors became available after this study was completed. All of the currently available inhibitors of Rho kinase (Y-27632[38], hydroxyfasudil[39], HA1077[49]) can be used *in vivo*. This provides a further focus on the role of downstream targets of Rho. One aspect that requires special care under these conditions, however, is that Rho proteins could interfere with the regulation of vessel tone, but this is possibly only the case in hypertension.[38] Besides investigating endothelial hyperpermeability

under inflammatory conditions it is desirable to study the large vessel hyperpermeability involved, that occurs in atherosclerotic vessels and in particular in areas treated with an expandable stent.[42] Several well-characterized mouse models that develop human-like atherosclerosis are currently available and could be used to study the contribution of endothelial hyperpermeability to the development of atherosclerosis and the mechanisms involved, e.g. the Apo E3 Leiden mouse. In chapter seven we provided evidence that Rho/Rho kinase-signaling is also activated by VEGF. Investigation of a possible involvement of Rho/Rho kinase in VEGF-induced vascular leakage *in vivo* merits attention, as multiple forms of vascular leakage are triggered by VEGF, e.g. vascular leakage associated with angiogenesis (capillaries) and the hyperpermeability of the tumor vasculature.

Another aspect that requires further investigation is the role of cAMP in barrier integrity.[50; 51] Besides treatment with antihistaminergic agents elevation of intracellular cAMP levels currently is one of the few ways known to improve endothelial barrier function. The precise mechanism of action of cAMP is still poorly understood, but involves the activation of the cAMP-dependent kinase[52], better known as PKA, and attenuation of MLC phosphorylation.[53; 54] A highly interesting finding in this context is the discovery that RhoA is a substrate for PKA.[55; 56] Phosphorylation of RhoA by PKA decreased guanine-nucleotide exchange activity of RhoA and thus decreased Rho function. This might explain multiple aspects of the effects of elevation of intracellular cAMP levels. Elevation of cAMP improves basal endothelial permeability, and also prevents the thrombin-induced increase in permeability. Both under basal and thrombin-stimulated conditions inhibition of Rho-activity lowers endothelial permeability. Therefore, it seems possible that cAMP could improve endothelial integrity by inhibition of Rho.

A third item, which is underestimated, is the role of protein phosphatases in the regulation of endothelial barrier function. In the last few years it has become clear that the activity of phosphatases is not a steady one at all, but is actively regulated. Protein phosphatase activity is expected to be especially of importance for improving the barrier function, as protein phosphorylations highly contribute to barrier disruption. For one protein phosphatase there is firm evidence for the involvement in the regulation of endothelial permeability. Several reports indicated that thrombin inhibits endothelial myosin phosphatase activity via Rho and Rho kinase and that the inhibition of the myosin phosphatase contributes to the thrombin-induced hyperpermeability.[1; 57; 58] Our finding on the important role of PTK in the regulation of endothelial barrier function demonstrates that other phosphorylations than the MLC phosphorylation are involved. Therefore, it is also highly likely that other

phosphatase activities than the myosine phosphatase activity contribute to the recovery of a disrupted barrier function.

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NEDERLANDSE SAMENVATTING

Het in dit proefschrift beschreven onderzoek heeft zich gericht op de mechanismen die ten grondslag liggen aan langdurige bloedvatlekkage. De binnenwandbekleding van de bloedvaten, die endotheel genoemd wordt, vormt de belangrijkste barrière die de uittreding (extravasatie) van stoffen uit het bloed naar de omringende weefsels actief reguleert. Onder omstandigheden dat het endotheel geactiveerd (of beschadigd) is, kan de barrière functie van het endotheel ernstig verstoord zijn. Dit kan leiden tot de ongewenste uittreding van vocht uit de bloedbaan en oedeem vorming, met als gevolg schade aan weefsels en organen. Het onderzoek beschreven in dit proefschrift heeft geleid tot meer inzicht in de regulatie van de endotheel barrière functie. De essentie hiervan ligt in de bevinding dat de vasoactieve stoffen histamine en trombine op een verschillende wijze de endotheel permeabiliteit (= doorlaatbaarheid) doen toenemen. We zijn nu in staat om de kortdurende verandering in endotheel permeabiliteit, die ook in de microcirculatie optreedt na toedienen van een vasoactieve stof aan een gezonde postcapillaire venule (= een klein bloedvat, dat zich op die plaats van het vaatbed bevindt, waar vaatlekkage optreedt bij ontstekingen), na te bootsen door endotheelcellen afkomstig uit de venen (aders) van menselijke navelstrengen gekweekt op poreuze filters in serum-vrij medium te stimuleren met histamine. Er treedt dan een toename op in de endotheel permeabiliteit die slechts enkele minuten aanhoudt, waarna de barrièrefunctie weer volledig herstelt. Deze situatie is vergelijkbaar met de reaktie die optreedt na een muggenbeet: er ontstaat een kleine lokale zwelling. Wanneer gekweekte endotheel monolagen echter met trombine gestimuleerd worden, treedt een langdurige verhoging van de endotheel permeabiliteit op, die minimaal een uur aanhoudt. Dit heeft ons in staat gesteld om specifiek mechanismen te bestuderen die betrokken zijn bij het ontstaan van langdurig verhoogde permeabiliteit. Dit met het doel een betere behandeling dichterbij te brengen van patiënten met langdurige vaatlekkage, die -als ze hart of longen treft- levensbedreigend kan zijn.

De oorspronkelijke hoofdvraag van het onderzoek was welke processen in het endotheel, naast de instroom van calcium, een rol zouden kunnen spelen in de langdurige verhoging van de endotheel permeabiliteit en op welke wijze in het endotheel de fosforylering van de regulatoire myosine lichte keten (MLC) gereguleerd is (zie volgende alinea). In retrospect is de vraagstelling een hele juiste gebleken, zij het met een andere als de verwachte uitkomst.

Bij zowel de respons op histamine als die op trombine speelt de fosforylering van de MLC een belangrijke rol. Analoog aan de veranderingen in endotheel permeabiliteit induceert histamine een transiënte verhoging in de MLC fosforylering en trombine een meer

langdurende verhoging. Fosforylering van de MLC induceert een conformatie verandering in het myosine molekuul, die resulteert in een toegenomen interactie tussen myosine en actine molekulen en een daarmee gepaard gaand contractie (=samentrekking) proces. Dit contractie proces speelt zich af aan de randen van de endotheelcel. Hierdoor ontstaan er minitieuse gaatjes tussen de endotheelcellen, die de permeabiliteit van de endotheellaag doen toenemen en passage van macromoleculen over de endotheel monolaag toestaan. Dit contractieproces is heel vergelijkbaar met het samentrekken (=contraheren) van gladde spiercellen, zij het dat er wel verschillen zijn aan te wijzen in de eiwitten die betrokken zijn bij de contractie van de endotheelcel en de gladde spiercel. Teneinde de rol van de MLC fosforylering in de regulatie van de permeabiliteit nader te kunnen bestuderen werd een 2-dimensionaal gel electrophorese systeem opgezet (i.s.m. prof Drenckhahn, Würzburg). In een later stadium is een nieuwe, 1-dimensionale methode om MLC fosforylering te meten opgezet, die minder bewerkelijk is en het mogelijk maakt om meerdere monsters te gelijkertijd te meten. We zijn ons gaan realiseren dat de MLC fosforylering op verschillende manieren aangestuurd kan worden. Zo komen in endotheel behalve de klassieke MLC kinase (= het eiwit dat de MLC fosforyleert) ook een endotheel specifieke MLC kinase en een zogenaamd embryonaal MLC kinase voor. Voorts zou Rho kinase ook als een MLC kinase kunnen werken. Wat betreft expressie van al deze kinasen werd geen verschil gevonden tussen de verschillende typen gekweekt endotheel.

Geheel conform het klassieke idee bleek histamine de permeabiliteit te verhogen door activering van de MLC kinase via calcium en calmoduline. De trombine respons was slechts partiëel te blokkeren met remmers van calcium, calmoduline en de MLC kinase. Dit suggereert dat andere of tenminste additionele mechanismen betrokken zijn bij de trombine respons. Histamine en trombine induceren een identieke toename in intracellulair calcium in het endotheel, zodat het verschil op het nivo van calcium tussen beide responsen niet waarschijnlijk is. Ook activering van protein kinase C -hoewel bekend in dierlijk endotheel van belang te zijn voor regulatie van barrièrefunctie- speelt geen majeure rol in de regulatie de humane endotheel barrièrefunctie, daar remmers van protein kinase C –gebruikt werden Ro31-8220 en calphostin C- geen effect hadden op de trombine respons.

De hoofdbevinding van ons onderzoek is dat trombine de permeabiliteit verhoogt door naast calcium, het kleine GTPase RhoA en tyrosine kinasen te activeren. Deze 3 processen blijken (deels) als onafhankelijke routes te opereren (zie figuur).

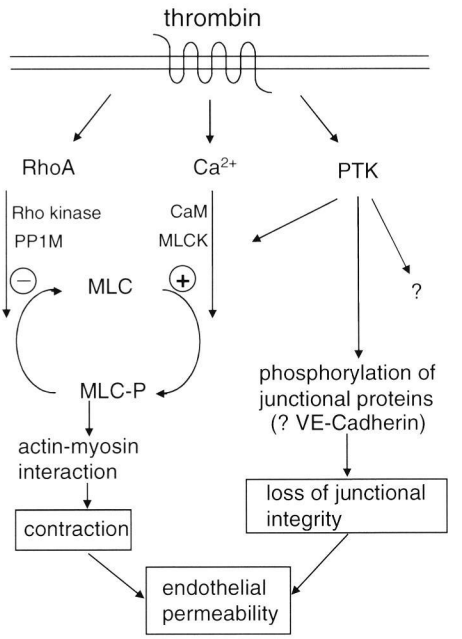
Actine kleuringen van endotheelcellen gestimuleerd met trombine lieten enorme veranderingen in het actine cytoskelet zien, waarbij met name de vorming van lange actine kabels door de cel heen, de zogenaamde stress fibers, opviel. *In vivo* treden stress fibers op

die plaatsen in het vaatbed op die gevoelig zijn voor het ontstaan van atherosclerostische plaques. Uit studies in fibroblasten en gladde spiercellen was bekend dat de kleine GTPase RhoA een belangrijke rol speelt in de vorming van deze structuren. Remming van RhoA in endotheel met C3 transferase –een toxine uit *Clostridium botulinium*- reduceerde niet alleen de door trombine geïnduceerde veranderingen in het cytoskelet, maar ook de verhoogde endotheel permeabiliteit en de vergezelde MLC fosforylering. In samenwerking met Dr. Collard van het Nederlands Kanker Instituut werd aangetoond dat trombine RhoA ook daadwerkelijk activeert in endotheel. Gebruik makend van een recent ontwikkelde remmer van het Rho-afhankelijke kinase, afkomstig van de Japanse firma Yoshitomi, werd aangetoond dat al deze effecten van RhoA via Rho kinase verlopen. Behalve de MLC kinase activiteit die Rho kinase in de reageerbuis heeft, remt Rho kinase de myosine fosfatase. Op deze wijze resulteert de gelijktijdige transiënte activering van de MLC kinase en de remming myosine fosfatase door trombine in een meer langdurende verhoging van de MLC fosforylering en in verhoogde permeabiliteit. RhoA bleek niet betrokken te zijn bij de transiënte histamine respons.

Het belang van dit verworven inzicht wordt onderstreept door onze bevinding dat de hyperpermeabiliteit verminderd wordt door de endotheelcellen voor te behandelen met het medicijn simvastatine. Simvastatine behoort tot een klasse van veel gebruikte farmaca in de behandeling van patiënten met hypercholesterolemie (= het hebben van een te hoge concentratie cholesterol in het bloed, zie ook de sectie 'Klinische relevantie') en verbetert de endotheel barrière functie mogelijk via inactivering van RhoA.

Met behulp van de tyrosine kinase remmer genisteïne werd aangetoond dat ook tyrosine fosforyleringen betrokken zijn bij de trombine respons. De remming van de trombine respons door genisteïne was zowel additief aan remming door het wegvangen van intracellulair calcium als aan remming van Rho kinase en had geen effect op de activering van RhoA. Dit geeft aan dat het 3 verschillende manieren betreft waarop endotheelhyperpermeabiliteit geïnduceerd kan worden. Welke tyrosine kinasen betrokken zijn bij de trombine respons is nog onbekend. Gebruik van andere remmers liet wel een zekere specifiteit zien. Zo had herbymicine A een vergelijkbaar effect met genisteïne en had de tyrosine kinase remmer tyrphostin A47 helemaal geen effect. Recentelijk is aangetoond dat tyrosine fosforylering van adherens junction eiwitten resulteert in disruptie van cel-cel interacties en betrokken is bij de vorming van de kleine gaatjes tussen de endotheel cellen als gevolg van blootstelling aan vasoactieve stoffen. Remming van tyrosine kinasen had geen effect op de histamine respons.

Behalve trombine zijn ook andere vasoactieve stoffen onderzocht, die de permeabiliteit zouden kunnen verhogen. Hierbij hebben we LPA (lysophosphatidic acid) geïdentificeerd als een tot nu toe onbekende mediator die permeabiliteit doet toenemen via activering van RhoA. Deze bevinding is van belang omdat recent is aangetoond dat LPA een van de componenten uit geoxideerd LDL is, die bijdraagt aan de activering van het endotheel door geoxideerd LDL. Geoxideerd LDL is een belangrijke risicofactor voor het ontstaan van atherosclerose ('aderverkalking'). Door verhoging van de endotheel permeabiliteit zou LPA bij kunnen dragen aan de ophoping van geoxideerd LDL in de vaatwand en daarmee aan de ontwikkeling van atherosclerose. LPA is een klassieke activator van RhoA. In gekweekt endotheel ging activering van RhoA niet gepaard met een verandering in intracellulair calcium. Dit impliceert dat activering van RhoA en instroom van calcium 2 onafhankelijke routes zijn, die permeabiliteit verhogen.



Hypothetisch schema dat de rol van calcium, RhoA/Rho kinase en tyrosine fosforyleringen in verhoogde endotheel permeabiliteit illustreert. CaM staat voor calmoduline; MLC voor myosine lichte keten; MLCK voor MLC kinase; MLC-P voor gefosforyleerd MLC; PP1M voor myosine phosphatase type 1; en PTK voor protein tyrosine kinase.

Naast de vasoactieve stof LPA hebben we ook onderzoek gedaan aan de VEGF. VEGF staat voor vascular endothelial growth factor en is een groeifactor, die de vorming van nieuwe bloedvaatjes stimuleert (=angiogenese). Deze angiogene factor VEGF is in eerste instantie ontdekt als Vascular Permeability Factor. Meer en meer is het inzicht ontstaan dat verhoogde endotheel permeabiliteit en de vorming van nieuwe bloedvaatjes geen 2 afzonderlijke processen zijn, maar in elkaars verlengde liggen. Nauwe samenhang bleek al uit het gegeven dat angiogenese vrijwel altijd gepaard gaat met een toename in de permeabiliteit. Verhoogde permeabiliteit resulteert in de uittreding van een fibrineus exudaat. Dit fibrineuze exudaat vormt een uitstekende tijdelijke matrix voor de ingroei van nieuwe bloedvaatjes. Onze bevinding dat Rho kinase, behalve bij verhoogde permeabiliteit, ook betrokken is bij de door VEGF geïnduceerde cytoskeletaire veranderingen en de angiogenese geven aan dit inzicht nu ook mechanistische onderbouwing.

In conclusie, het in dit proefschrift beschreven onderzoek laat zien dat de endotheel permeabiliteit op verschillende manieren verhoogd kan worden. Instroom van calcium geeft een kortdurende verhoging van de endotheel permeabiliteit, terwijl activering van RhoA en tyrosine kinasen leidt tot langdurige veranderingen van de permeabiliteit. Deze bevindingen geven nieuwe aanknopingspunten voor de ontwikkeling van farmaca om langdurende vaatlekkage te bestrijden.

Klinische relevantie

Zoals hiervoor is aangegeven, heeft dit onderzoek duidelijk gemaakt dat aan transiënt en langdurig verhoogde endotheel permeabiliteit verschillende mechanismen ten grondslag liggen. Deze verworven inzichten kunnen direct bij gaan dragen aan de behandeling van het vaatlekkageprobleem, dat regelmatig bij patiënten met hart en vaatziekten optreedt. Huidige therapieën om een gestoorde endotheel barrièrefunctie te verbeteren -antihistaminica en cAMP verhogende farmaca/ß-adrenergica- falen als het gaat om de behandeling van langdurige vaatlekkage. In het eerste geval wordt alleen de instroom van calcium geremd, maar niet de activering van RhoA/Rho kinase en tyrosine kinasen, die juist een belangrijke rol spelen in het ontwikkelen van langdurige verhoging van de endotheel permeabiliteit. In het laatste geval treedt gewenning/desensitisatie aan de medicatie op, zodat behandeling slechts tijdelijk resulteert in herstel van de verstoorde endotheel barrière functie.

Een eerste ontwikkeling die van belang is voor het behandelen van vaatlekkage is het

beschikbaar komen van een specifieke remmer van Rho kinase met de codenaam Y-27632. In ons permeabiliteitsmodel verlaagt de Rho kinase remmer de trombine geïnduceerde permeabiliteit met ongeveer 50%. Van deze remmer is verder bekend dat zij ook effectief is in het remmen van hypercontractie processen in gladde spiercellen en bloeddruk verlagend werkt in verschillende diermodellen voor hypertensie. Deze verbinding heeft voor zover bekend geen nadelige invloed op normale bloeddruk, hartfunctie en bloed- en urine chemie. Klinische toepassing in de behandeling van het vaatlekkageprobleem lijkt daarom ook haalbaar.

Een minstens zo belangrijke ontwikkeling voor de behandeling van vaatlekkage is onze bevinding dat simvastatine een gestoorde endotheelbarrière functie kan verbeteren, via inactivering van RhoA. Zowel in ons *in vitro* model als *in vivo* in aorta's van atherosclerotische konijnen had behandeling met simvastatine een gunstig resultaat. Met statines is veel klinische ervaring opgedaan in de behandeling van hypercholesterolemische patienten. Statines worden algemeen als veilig beschouwd. Verbetering van endotheel (barrière) functie draagt waarschijnlijk bij aan de positieve effecten van statines en zou mogelijk ook de vaatlekkage kunnen remmen als het gevolg van het plaatsen van stents. Behalve in de grote vaten, is effectiviteit van statines in de vermindering van vaatlekkage in de postcapillaire venules, zoals die veelvuldig optreedt in acute ontstekingen, waarschijnlijk. Andere onderzoekers hebben namelijk recent aangetoond dat simvastatine ook in de microvasculatuur effectief is en daar de endotheel-leukocyte (= witte bloedcel) interactie remt.

Op de langere termijn lijkt klinische toepassing van onze bevinding dat tyrosine fosforyleringen bijdragen aan barrièredysfunctie reëel en zeker zo veelbelovend als remming van RhoA/Rho kinase. Activering van tyrosine kinasen blijkt namelijk een heel algemeen mechanisme van verhoogde permeabiliteit te zijn en is ook betrokken permeabiliteitsveranderingen als gevolg van blootstelling aan stoffen als bijv. VEGF, waterstofperoxide en endotoxine. De ontwikkeling van specifieke farmaca gericht op remming van deze tyrosine fosforylering wordt echter nog gehinderd, doordat de identiteit van de betrokken tyrosine kinasen niet bekend is. De verwachting is dat bij het herstel van de gestoorde barrièrefunctie tyrosine defosforyleringen door specifieke tyrosine fosfatasen betrokken zijn. Toekomstig onderzoek zal zich dan ook richten op het karakteriseren en identificeren van tyrosine kinasen en fosfatasen, die betrokken zijn bij veranderingen in endotheel permeabiliteit.

ABBREVIATIONS

ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N',-tetraacetic acid
bFGF	basic fibroblast growth factor
$[Ca^{2+}]_i$	Intracellular calcium concentration
CaM	calmodulin
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophsophate
CNF1	cytotoxic necrotizing factor-1
CRIB	Cdc42/rac1 Interactive Binding domain
DAG	diacyl glycerol
EC	endothelial cell
ECGF	endothelial cell growth factor
eNOS	endothelial nitric oxide synthase
ERM	ezrin/radixin/moesin
FA	focal adhesion
F-actin	filamentous actin
FAK	focal adhesion kinase
FITC	fluorescein thiocyanate
fMLP	formylMet-Leu-Phe
GAP	GTPase-activating protein
GDI	guanine dissociation inhibitor
GEF	guanine nucleotide exchange factor
GPCR	G protein coupled receptor
G protein	GTP-binding protein
GST	gluthathione-S-transferase
GTP	guanosine triphosphate
HAEC	human aortic endothelial cell
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
hMVEC	human foreskin microvascular endothelial cell
HRP	horseradish peroxidase
HSA	human serum albumin
HUVEC	human umbilical cord endothelial cell

IBMX	isobutyl methylxanthine
IP_3	inositol triphosphate
kD	kilo Dalton
LDL	low density lipoprotein
LPA	lysophosphatidic acid
LPS	lipopolysaccharide
MBS	myosin binding subunit of myosin phosphatase
MLC	myosin light-chain
MLCK	myosin light chain kinase
MRCK	myotonic dystrophy vkinase-related Cdc42-binding kinase
NBCS	newborn calf serum
NO	nitric oxide
PAF	platelet activating factor
Pak	P21-activated kinase
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate-13-acetate
PMT	*Pasteurella multocida* toxin
PP1M	myosin phophatase type 1
PTK	protein tyrosine kinase
RBD	Rho-binding domain
SEM	standard error of the mean
SF	stress fiber
SMC	smooth muscle cell
S1P	sphingosine 1-phosphate
TEER	transendothelial electrical resistance
TFP	trifluoroperazine
TNF	tumor necrosis factor
TRAP	thrombin receptor-activating peptide
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein
VPF	vascular permeability factor

LIST OF PUBLICATIONS

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CURRICULUM VITAE

Geerten van Nieuw Amerongen was born on October 7th 1971 in Breukelen, The Netherlands. Undergraduate education was received at the Driestar College (grammar school) in Gouda from 1984 till 1990. He studied Chemistry (Specialization: Biochemistry and Molecular Biology) and Pharmacochemistry (Specialization: Molecular Pharmacology) at the Vrije Universiteit, Amsterdam. From 1995-1999 he was a Ph.D. student under supervision of Prof.dr.V.W.M. van Hinsbergh at the Dept. of Internal Medicine of the Rijksuniversiteit, Leiden, posted to the Dept. of Vascular and Connective tissue Research, TNO-Prevention & Health, Leiden. During this period he conducted the studies presented in this thesis. His main interest was the regulation of endothelial barrier function. A major contribution of the RhoA/Rho kinase signal transduction pathway in prolonged endothelial barrier hyperpermeability was demonstrated. For this work he received a Young Investigator Award from the German Society for Histochemistry in 1998. Subsequently, in 1999 he was appointed at the Department of Physiology, Faculty of Medicine, Vrije Universiteit Amsterdam, to continue the studies described in this thesis. Currently, as a part of this project he spends one year in the Cardiovascular Center of the University of Rochester NY (Prof.dr.B. Berk) to study the role of tyrosine (de)phosphorylations in the signal transduction of the vascular wall.

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Des HEEREN werken zijn zeer groot,
Wie ooit daarin zijn lust genoot,
Doorzoekt die ijv'rig en bestendig.

Psalm 111:2 Johannes Eusebius Voet 1773