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Role of RhoA and Rho Kinase in Lysophosphatidic Acid–Induced Endothelial Barrier Dysfunction

Geerten P. van Nieuw Amerongen, Mario A. Vermeer, Victor W.M. van Hinsbergh

***Abstract*—**In the present study, the roles of the small GTPase RhoA and its target Rho kinase in endothelial permeability were investigated in vitro. We have shown previously that, in addition to a rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), 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 lysophosphatidic acid (LPA), a commonly used RhoA activator. LPA induced a 2- to 3-fold increase in the passage of horseradish peroxidase (HRP) across endothelial monolayers that lasted for several hours, whereas 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 and the formation of focal attachment sites. LPA induced only a transient increase in myosin light-chain (MLC) phosphorylation, which returned to basal level within 10 minutes. In endothelial cells, $[Ca^{2+}]_i$ was not elevated by LPA. Chelation of $Ca^{2+}{}_i$ ions by 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid did not prevent the LPA-induced passage of HRP. Apparently, a low degree of MLC kinase activation occurred, because the MLC kinase inhibitor KT5926 reduced the levels of both basal and LPA-stimulated HRP passage. Inhibition of RhoA by the C3 transferase from *Clostridium botulinum* inhibited the LPA-induced cytoskeletal changes and prevented the LPA-induced HRP passage. Inhibition of Rho kinase by Y-27632 completely prevented the LPA-induced increase in HRP passage without affecting basal permeability. These data indicate that LPA-induced endothelial hyperpermeability occurs without a change in $[Ca^{2+}]_i$ and requires activation of RhoA and Rho kinase. **(*Arterioscler Thromb Vasc Biol*. 2000;20:e127-e133.)**

Key Words: human endothelial cells ■ RhoA ■ calcium ■ myosin light-chain phosphorylation ■ myosin light-chain kinase

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 (ECs), 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 myosin light chain (MLC) by the Ca^{2+}/calmodulin-dependent kinase I, the classic MLC kinase, directs the actin-myosin–based contraction process in ECs and is dependent on calcium ions and calmodulin.[1]

In the past 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,[2] an enzyme that is activated by the small GTPases Cdc42 and Rac, reduces MLC kinase activity, whereas in ECs, p21-activated kinase increases MLC phosphorylation.[3] The small GTPase RhoA, in addition to an elevation of the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, is involved in the prolonged endothelial barrier dysfunction and elevated MLC phosphorylation levels induced by thrombin.[4,5] Soluble factors from serum such as lysophosphatidic acid (LPA) and sphingosine 1-phosphate are thought to activate RhoA.[6,7] To address the question of whether activation of RhoA per se induces prolonged endothelial barrier dysfunction, we studied the effects of LPA on endothelial barrier function and investigated the mechanisms by which RhoA can induce endothelial permeability.

The phospholipid LPA is known to be formed by and released from activated platelets and can be generated by the action of secretory phospholipase A_2. Interestingly, Siess et al[8] have recently shown that mild oxidation of LDL also generates biologically active LPA, thus stimulating platelet activation and endothelial stress-fiber formation. LPA binds to the Edg subfamily of high-affinity, G protein–coupled heptahelical receptors, by which it can activate various second messengers, including RhoA, via $G_{12/13}$.[9] When RhoA is activated, GDP is exchanged for GTP. Activation of RhoA can be inhibited by the *Clostridium botulinum* C3 transferase toxin, which specifically ADP-ribosylates RhoA at Asn41.[10,11] In its GTP-bound form, RhoA interacts with

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several downstream targets, such as protein kinase N, Rho kinase, rhotekin, rhophilin, phosphatidylinositol 4-phosphate 5-kinase, citron, and p140mDia (reviewed in References 12 through 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[17,18] and possibly, by direct MLC phosphorylation.[19] Uehata et al[20] and Ishizaki et al[21] recently described a synthetic pyridine analogue that inhibits Rho kinase with high specificity compared with MLC kinase. This cell-permeant inhibitor, Y-27632, was able to prevent Rho-mediated stress-fiber formation and smooth muscle contraction. In the present study, we investigated the effects of LPA-induced RhoA activation on EC permeability and used Y-27632 to demonstrate the involvement of Rho kinase in the LPA-enhanced endothelial permeability.

Methods

Materials

Tissue-culture plasticware and Transwells (diameter 0.65 cm, pore size 3 μm) were obtained from Costar; cell-culture reagents used were as previously described.[4] Bovine thrombin was from Leo Pharmaceutical Products. Horseradish peroxidase (HRP), LPA (stored under N_2), and anti-vinculin immunoglobulin were obtained from Sigma Chemical Co.[22] 1,2-bis(2-Aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) and rhodamine-phalloidin were from Molecular Probes. Pyrogen-free human serum albumin (HSA) was from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands). C3 transferase toxin was kindly provided by Dr A. Ridley (Ludwig Institute, London, UK). [^{32}P]orthophosphoric acid, Tran^{35}*S* label, and KT5926 were from ICN Pharmaceuticals, Inc. Tritiated LPA (specific activity, 30 to 60 Ci/mmol) was from NEN Life Science Products, Inc. Anti-platelet myosin immunoglobulin (nonmuscle) was from Sanbio; rabbit anti-mouse IgG-FITC was from Dakopatts. Y-27632 was supplied by Welfide Corporation.

Cell Culture and Evaluation of Barrier Function

Human umbilical vein ECs were isolated and cultured as previously indicated.[23,24] One hour before stimulation with LPA, confluent endothelial monolayers were deprived of serum and kept in medium 199 with 1% HSA. Unless indicated otherwise, ECs were stimulated with 13 μmol/L LPA. Based on the dilution of LPA, 20 μmol/L was expected to be added. Because LPA binds to culture plasticware, the actual LPA concentration was determined by using tritiated LPA to monitor its fate during dilution. Instead of 20 μmol/L, the actual concentration during incubation was 13.1±1.8 μmol/L (mean±SD, n=6). Values of LPA concentrations are given after correction for tritiated LPA recovery. Barrier function was evaluated by the transfer of HRP across human umbilical vein EC monolayers grown on fibronectin-coated polycarbonate filters of the Transwell system.[4]

MLC Phosphorylation

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

Extraction and Assay of Intracellular cAMP and $[Ca^{2+}]_i$

Intracellular cAMP levels were determined by radioimmunoassay (Amersham) as described previously.[4,23] $[Ca^{2+}]_i$ levels were determined by the fura 2 method as indicated previously.[23] As a positive control, cells were permeabilized with 10 μmol/L ionomycin, and afterward, the Ca^{2+} ions were displaced from fura 2 by incubation with 1 mmol/L $MnCl_2$.

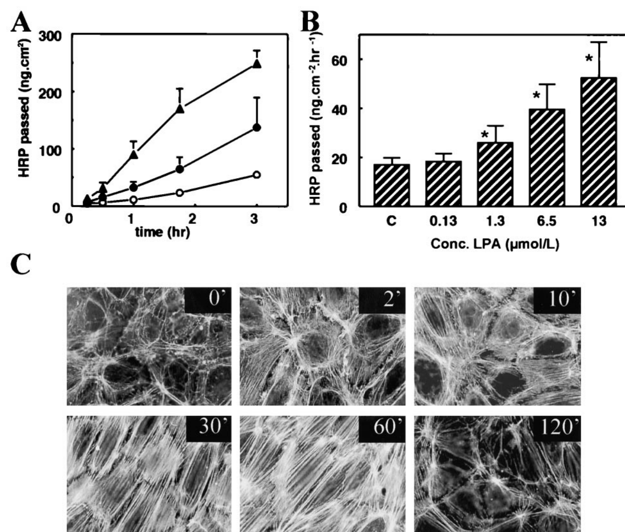

Figure I. Endothelial permeability is increased by LPA in a time- and concentration-dependent manner and is accompanied by changes in the F-actin cytoskeleton. A, Time curve of LPA-induced HRP passage across endothelial monolayers. Endothelial monolayers were stimulated with 13 μmol/L LPA (●) or 1 U/mL thrombin (▲) or were incubated without stimulation (○), and passage of HRP molecules was measured as described in Methods. Values are mean±SEM of 6 filter cultures in triplicate. *$P<0.05$, cells that were stimulated with LPA vs sham-treated cells. B, Concentration curve of LPA-induced HRP passage across endothelial monolayers. HRP passage was determined 1 hour after sham treatment (C) or exposure to the indicated concentrations of LPA. Values are mean±SEM of 6 filter cultures in triplicate. C, LPA induced changes in the F-actin cytoskeleton. ECs were stimulated with 13 μmol/L LPA, fixed at the time points indicated, and stained for F-actin with rhodamine-phalloidin. Similar results were observed in 4 different cultures.

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±SEM. Comparisons between >2 groups were made by 1-way ANOVA, followed by a Bonferroni-adjusted χ^2 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 HRP (filled circles in Figure I compared with basal HRP passage, as indicated by open circles). Comparable to the thrombin-induced increase in HRP passage (filled triangles in Figure I-A), 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 with the 5- to 10-fold increase in permeability that is usually observed after stimulation by thrombin and was just doubled after 1 hour (43.9±8.7 versus 21.4±4.6 ng · cm^{-2} · h^{-1} in unstimulated monolayers; mean±SEM of 9 cultures in triplicate; $P<0.001$). The effect of LPA on barrier

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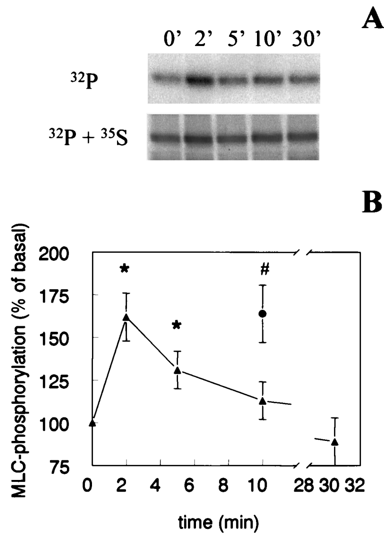

Figure II. MLC phosphorylation is stimulated transiently by LPA. A, Autoradiograph of MLCs immunoprecipitated from cells under basal conditions (0 minutes) and 2, 5, 10, and 30 minutes after stimulation with 13 μmol/L LPA. Cells were labeled with ^{32}P and ^{35}S as described previously.[4] Top, Exposure in which a filter was present to block the ^{35}S signal. Bottom, Exposure without a filter. B, Quantification of MLC phosphorylation. LPA (▲) significantly increased MLC phosphorylation at 2 and 5 minutes. *$P<0.05$, LPA-stimulated vs nonstimulated cells. Values are 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/mL thrombin (●) on MLC phosphorylation at 10 minutes is shown. #$P<0.05$, thrombin stimulated vs nonstimulated cells.

function was concentration dependent and started in the low micromolar range (Figure I-B).

The LPA-induced change in endothelial permeability was accompanied by changes in the F-actin cytoskeleton. In control ECs, F-actin filaments were organized in a cortical network (Figure I-C). Two minutes after activation with LPA, short F-actin filaments were formed. After 10 minutes, the F-actin content was further increased; thin fibers formed and small gaps between the cells became visible. The number of F-actin fibers was maximal at 30 and 60 minutes. After 120 minutes, stress fibers disappeared completely.

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 II). In contrast, after a 10-minute stimulation by thrombin, MLC phosphorylation levels were still elevated (163±17% compared with basal levels; mean±SEM of 3 cultures in duplicate), and MLC phosphorylation remained elevated for at least 30 minutes, as was shown previously (see the inset to Figure 5 in Reference 4).

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.[2] We investigated whether

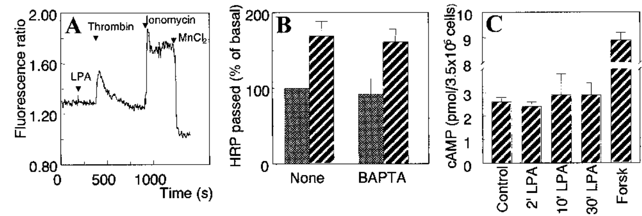

Figure III. $[Ca^{2+}]_i$ and cAMP levels are not involved in LPA-induced endothelial barrier dysfunction. A, Representative tracing of changes in cell calcium in human umbilical vein ECs exposed to LPA (13 μmol/L) and thrombin (1 U/mL). Cells were loaded with fura 2-AM, and $[Ca^{2+}]_i$ was monitored as described in 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 1 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 mean±SEM of 2 different cultures in triplicate. C, Effect of LPA (13 μmol/L) on cAMP content. Human umbilical vein ECs were preincubated for 1 hour in medium 199 with 1% HSA and stimulated for the times indicated with LPA (13 μmol/L) or vehicle. Fifteen minutes before stimulation, cells were preincubated with isobutyl methylxanthine (1 mmol/L). Forskolin (Forsk, 10 μmol/L) was used as a positive control for cAMP elevation. Data are mean±SEM of 3 different cultures in duplicate.

LPA could also induce a rise in $[Ca^{2+}]_i$ in human umbilical vein ECs, as was shown in a variety of cell types,[28] although those authors reported that several cell types lacked this Ca^{2+} response. We found that LPA did not elevate $[Ca^{2+}]_i$ (Figure III-A), whereas thrombin induced a transient rise in $[Ca^{2+}]_i$ in the same EC monolayers. To further exclude a role for Ca^{2+} ions in the LPA-induced passage of HRP molecules, we pretreated the ECs with the intracellular Ca^{2+} chelator BAPTA. Preincubation with 3 μmol/L BAPTA did not affect the LPA-induced passage of HRP (Figure III-B), whereas BAPTA completely blocked the Ca^{2+}-dependent increase in permeability abetted by histamine (see Figure 1D in Reference 4).

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

RhoA Is Involved in LPA-Induced Cytoskeletal Reorganization and Hyperpermeability

To verify that the effects of LPA on EC barrier functions were mediated by RhoA, ECs were pretreated with an inhibitor of RhoA, the C3 transferase from *C botulinum,* and subsequently stained for F-actin and vinculin, a component of focal adhesion complexes. In confluent ECs, focal contact sites were hardly detectable (Figure IV-A). Vinculin appeared as a thin, peripheral band and a diffuse, cytoplasmic staining. Preincubation of the cells with the RhoA inhibitor C3 transferase (5 μg/mL for 24 hours) had no effect on vinculin localization (Figures IV-A and IV-D), but the cortical F-actin network disappeared (Figures IV-B and IV-E). The F-actin that remained appeared as a thin, peripheral band that colocalized with vinculin (Figures IV-C and IV-F). The LPA-induced cytoskeletal changes (Figures IV-G through

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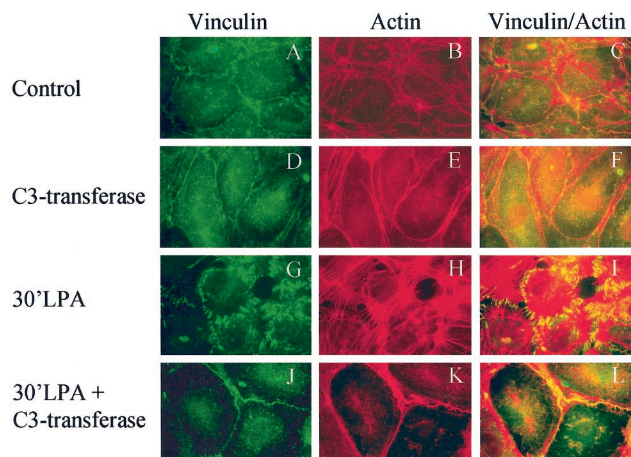

Figure IV. LPA-induced cytoskeletal reorganization is completely RhoA dependent. Immunocytochemical staining of vinculin (A, D, G, J), F-actin (B, E, H, K), or both (C, F, I, L). Human umbilical vein ECs were preincubated for 24 hours with 5 μg/mL C3 transferase and stimulated with 13 μmol/L LPA. Actin and vinculin were visualized as described in Methods. A–C, Basal condition; D–F, J–L, cells were preincubated with C3 transferase; G–L, cells were stimulated for 30 minutes with LPA. Similar results were observed in 3 different cultures.

IV-I) were completely dependent on the activation of RhoA, since preincubation of the cells with C3 transferase prevented the formation of focal adhesion complexes and an increase in F-actin filaments (Figures IV-J through IV-L). The cells remained flat.

Preincubation with C3 transferase also completely prevented the LPA-enhanced HRP passage (Figure V-A). Thus, LPA induced a dramatic change in the endothelial F-actin cytoskeleton in a RhoA-dependent manner, which was accompanied by a RhoA-dependent increase in endothelial permeability.

To investigate whether ongoing MLC kinase activity was also required for LPA-enhanced endothelial permeability, ECs were pretreated with the selective MLC kinase KT5926. KT5926 reduced basal permeability and completely prevented the LPA-enhanced HRP passage (Figure V-B).

Activation of Rho Kinase Is Essential for LPA-Enhanced Permeability

Recent data pointed to a role for Rho kinase, 1 of the targets of RhoA, in the formation of stress fibers and cell contrac-

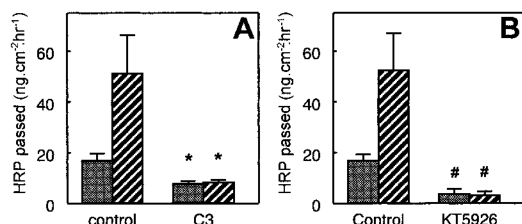

Figure V. Effect of C3 transferase and KT5926 on basal and LPA-enhanced permeability. Endothelial monolayers were pretreated for 24 hours with 5 μg/mL C3 transferase (A) or for 30 minutes with 10 μmol/L KT5926 (B). HRP passage was determined 1 hour after sham treatment (filled bars) or exposure to 13 μmol/L LPA (hatched bars). Values are mean±SEM of 6 filter cultures of 2 different donors. *$P<0.05$, cells that were pretreated with C3 transferase vs nonpretreated cells. #$P<0.05$, cells that were pretreated with KT5926 vs nonpretreated cells.

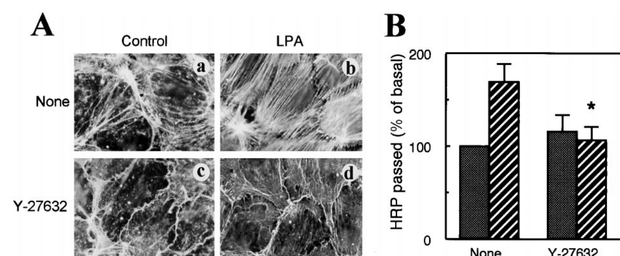

Figure VI. Effect of inhibition of Rho kinase on LPA-induced endothelial permeability. A, Immunocytochemical staining of F-actin. ECs were pretreated for 1 hour with 10 μmol/L Y-27632 (c, d) and stimulated for 30 minutes with 13 μmol/L LPA (b, d). Similar results were observed in 3 different cultures. B, Effect of preincubation with Y-27632 on basal (cross-hatched bars) and LPA-stimulated (hatched bars) HRP passage after 1 hour. Endothelial monolayers were preincubated for 1 hour with 10 μmol/L Y-27632. HRP passage is expressed as a percentage of basal HRP passage after 1 hour. Values are mean±SEM of 2 different cultures in triplicate. *$P<0.05$, cells that were pretreated vs nonpretreated cells.

tion.[19,21] 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 ECs 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 VI-A).

Subsequently, 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 VI-B) 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.

Discussion

In the present study, we have shown that activation of Rho by LPA is required to induce a prolonged increase in endothelial permeability in the absence of a rise in $[Ca^{2+}]_i$. 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 its role in the elevation in the number 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+}/calmodulin-dependent MLC phosphorylation, comparable to the calcium sensitization that is known from smooth muscle cells.[29] In the present study, the specific Rho activator LPA was used to study Rho-mediated mechanisms of endothelial permeability. In contrast to thrombin-enhanced endothelial permeability,[4] which is only partially dependent on the activation of RhoA, LPA-enhanced permeability was completely blocked by the inhibition of either Rho or Rho kinase.

Rather high concentrations of LPA were necessary to induce endothelial hyperpermeability. This may have resulted from partial desensitization of the LPA receptors. LPA is known to be present in serum in concentrations of up to

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2 μmol/L. Our cells were cultured in 20% serum and therefore exposed to a concentration of LPA that is in the range of reported receptor affinities.[9] Prolonged exposure to LPA results in downregulation of LPA receptors.[30] Thus, it is likely that the effect of LPA on endothelial barrier function is underestimated.

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 the EC monolayers that responded to thrombin with respect to Ca^{2+} mobilization. Second, chelation of $Ca^{2+}{}_i$ ions had no effect on the LPA-induced HRP passage. Thus, besides acting as a sensitizer for Ca^{2+}-induced changes in thrombin-enhanced endothelial permeability,[4] activation of RhoA induces endothelial permeability, even without a change in $[Ca^{2+}]_i$. This result agrees with the finding in a previous report in which Ca^{2+}-independent but Rho-dependent hyperpermeability induced by *Pasteurella multocida* toxin was suggested.[31] It should be noted that in ECs, MLC kinase is active at a low degree,[32] which explains why inhibition of MLC kinase by KT5926 reduced basal HRP passage. This basal MLC kinase activity was also required for the LPA-enhanced HRP passage, in agreement with previous reports that indicated that ongoing MLC kinase activity is involved in the cytoskeletal effects of LPA.[31,33,34] In fibroblasts, it has been shown that LPA lowers cAMP levels, probably by coupling G_I proteins to an LPA receptor,[35] and cAMP is known to improve endothelial barrier function.[25–27] However, LPA did not significantly lower cAMP levels in ECs, excluding the possibility of a reduction in 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 ECs by LPA.[36] In a recent report by those 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.[37] From these data, they suggested that LPA can actively regulate the opening of adherens junctions. Other studies do not favor a role for Rho proteins in the regulation of adherens junctions in ECs. Braga et al[38] showed that ECs 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 the inhibition of either Rho or Rac. This scenario fits with our finding that inhibition of RhoA and Rho kinase did not disrupt the cortical F-actin band, although it reduced the F-actin of ECs, and that inhibition of Rho kinase did not decrease basal endothelial barrier function. 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[39] showed that the Cdc42-, Rac-, and Rho-dependent TNF-α–induced stress-fiber formation was also accompanied, at least partly, by a 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 ECs. 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 ezrin/radixin/moesin family.[40] Recent data from Fukata et al[40] indicate that phosphorylation of adducin by Rho kinase activates the association of an F-actin–spectrin meshwork with the plasma membrane. Such a mechanism may affect the organization of cytoskeleton anchoring with the plasma membrane and junction complexes.

In bovine aortic endothelial monolayers, Alexander et al[41] observed an improvement of barrier function after stimulation with LPA. These apparently contrasting results are not caused by tissue-specific differences, because in our experience with human aortic endothelial monolayers, LPA decreased barrier function comparably to human umbilical vein EC monolayers (van Nieuw Amerongen et al, 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[17,18] or by its capacity to phosphorylate the MLC itself.[19] The former appears the most likely mechanism of Rho kinase–induced MLC phosphorylation in ECs, since it was shown that myosin phosphatase is inhibited by thrombin in ECs.[32,42,43]

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 sufficient to induce a prolonged EC contraction comparable to the latch-bridge state in smooth muscle cells, wherein maintenance of contraction occurs despite MLC dephosphorylation.[44]

A transient MLC phosphorylation does not necessarily lead to 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 finding 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[20] or other actions of Rho kinase on the cytoskeleton[45] contribute to this prolongation. However, the contribution of additional factors activated by LPA cannot be excluded. Phosphatidylinositol 4,5-biphosphate (PIP_2) may be involved, as intracellular levels of PIP_2 can be increased after activation of Rho[46] and PIP_2 is known to interfere with the actin cytoskeleton.[47] Furthermore, the activation of protein tyrosine kinases by LPA may also contribute to prolonged changes in the regulation of the cytoskeleton or cell-cell junctions.[48–52]

The increase in MLC phosphorylation precedes the appearance of stress fibers and focal adhesions. This sequence fits nicely with the model of Chrzanowska-Wodnicka and Burridge,[33] who showed that the Rho-induced MLC phosphorylation and tension development precede the assembly of stress fibers in fibroblast cells. The role of stress fibers in permeability is not precisely known and requires further investigation. They probably reflect actin–nonmuscle myosin interactions but do not necessarily per se contribute to a state

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of prolonged contraction of the ECs. Stress fibers obviously have contractile properties,[53] but on the other hand, they also stabilize ECs in regions of high shear.[34,54]

Our data point to an important role of Rho and Rho kinase in the regulation of endothelial permeability. Future studies will need to demonstrate if and when these factors are involved in altered endothelial barrier function in vivo. In large-vessel ECs, 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 ECs in vivo. However, it should be noted that Rho kinase plays a role in cell migration and that prolonged permeability might be a reflection of the altered behavior of ECs during cell migration and angiogenesis, which occur in wound healing and pathological conditions.

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