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During Gram-negative sepsis bacterial LPS induces endothelial cell contraction, actin reorganization, and loss of endothelial integrity by an unknown signal mechanism. In this study, we provide evidence that LPS-stimulation of endothelial cells (HUVEC) decreases myosin light chain (MLC) phosphatase, resulting in an increase in MLC phosphorylation followed by cell contraction. All of these LPS effects could be blocked by the Rho-GTPase inhibitor C3 transferase from Clostridium botulinum or the Rho kinase inhibitor Y-27632. These data suggest that LPS induces MLC phosphorylation via Rho/Rho kinase-mediated inhibition of MLC phosphatase in HUVEC. Furthermore, we observed that cAMP-elevating drugs, known to exert a vasoprotective function, mimicked the effects of C3 transferase and Y-27632, i.e., inhibited LPS-induced MLC phosphatase inactivation and MLC phosphorylation. cAMP elevation did not inhibit myosin phosphorylation induced by constitutively active V14Rho or the MLC phosphatase inhibitor calyculin and did not induce phosphorylation of RhoA in HUVEC, indicating inhibition of an upstream regulator of Rho/Rho kinase. Taken together, Rho/Rho kinase appears to be a central target for inflammatory mediators causing endothelial cell contraction such as bacterial toxins, but also for vasoprotective molecules elevating intracellular cAMP. The Journal of Immunology, 2000, 164: 6543–6549.

We recently identified an additional, Ca\textsuperscript{2+}-independent pathway by which thrombin and Pasteurella multocida toxin can control MLC phosphorylation in HUVEC. This pathway is mediated by the Ras-related GTP-binding protein Rho (3, 4). The ubiquitous Rho-GTPases play a pivotal role in the control of cellular actin rearrangements and cell shape (10). Depending on the cell type, Rho regulates formation of stress fibers and focal adhesion sites or cell contraction (3, 11–13). In its active GTP-bound form, Rho stimulates the effector molecule p160 Rho kinase which phosphorylates and thereby inactivates MLC phosphatase (14–16). It is assumed that Rho-mediated inactivation of MLC phosphatase favors MLC phosphorylation in the presence of MLC kinase activity (3, 4). Conversely, when Rho is inhibited, e.g., by C3 transferase, MLC phosphatase activity increases and MLC dephosphorylation is favored. Seemingly through this mechanism, thrombin- and P. multocida toxin-induced MLC phosphorylation and cell contraction can be blocked by Rho inhibitors.

It is well documented that drugs that elevate the intracellular concentration of cAMP prevent vascular leakage induced by inflammatory mediators (17–19). cAMP seems to inhibit thrombin- and histamine-induced MLC phosphorylation and endothelial cell contraction without affecting the Ca\textsuperscript{2+} influx induced by these mediators (7, 18). Elevation of cAMP was shown to disrupt the actin cytoskeleton of quiescent endothelial cells and to prevent thrombin- and inducible nitric oxide synthase-mediated cell contraction (20). Importantly, inhibiting Rho by C3 transferase from Clostridium botulinum blocks endothelial contractility in the same manner as cAMP-elevating drugs (3, 18). Moreover, cAMP-elevating agents and C3 transferase can in a similar way inhibit various Rho-mediated cell functions such as tyrosine phosphorylation of paxillin in murine Y1 adrenal cells (21), NK cell function (22), melanoma cell differentiation (23), and integrin receptor clustering (24). Furthermore, it was shown that cAMP-induced morphological changes of neuronal cells are counteracted by active Rho (25). Given these widespread similarities in the cellular effects of cAMP-elevating agents and Rho inhibitors, we reasoned that the inhibitory effect of cAMP...
on endothelial cell contraction might be due to inhibition of the Rho/Rho kinase signaling pathway. A molecular mechanism by which cAMP could directly inhibit Rho was suggested previously. It was shown that protein kinase A (PKA) can phosphorylate recombiant RhoA on serine 188 in vitro and in intact NK cells (22). However, so far it has not been demonstrated that PKA phosphorylates RhoA in all cell types.

In this study, we provide evidence that in human endothelial cells LPS induces MLC phosphatase deactivation and an increase in MLC phosphorylation via Rho/Rho kinase. In addition, we show that cAMP-elevating substances inhibit Rho/Rho kinase signaling at a level upstream of Rho, leading to a blockage of LPS-induced MLC phosphorylation.

Materials and Methods

LPS was phenol extracted from Escherichia coli (Sigma, Deisenhofen, Germany); Rho kinase inhibitor Y-27632 was kindly provided by Akiko Yoshimura (Yoshitomi Pharmaceuticals, Iruma-Shi-Saitama, Japan); and anti-phospho-MLC Ab was prepared as described previously (26). PKI-Yoshimura (Yoshitomi Pharmaceuticals, Iruma-Shi-Saitama, Japan); and Rho kinase inhibitor Y-27632 was kindly provided by Akiko Yoshimura (Yoshitomi Pharmaceuticals, Iruma-Shi-Saitama, Japan). For all experiments, cells were plated at a density of 2 x 10^4 cells/cm^2 and cultured on Cellocate glass coverslips (Eppendorf, Hamburg, Germany) plastic culture flasks and cultured in endothelial growth medium (Promo Cell, Heidelberg, Germany) containing endothelial cell growth supplement/heparin and 10% FCS. For all experiments, cells were plated at a density of 2 x 10^4 cells/cm^2 and grown to confluency for 10 days, with medium changes every 2–3 days.

Cell culture

HUVEC were obtained and cultured as described previously (3, 4). Briefly, cells harvested from umbilical cords were plated onto collagen-coated (24 h, 100 μg/ml collagen G; Biochrom, Berlin, Germany) plastic culture flasks and cultured in endothelial growth medium (Promo Cell, Heidelberg, Germany) containing endothelial cell growth supplement/heparin and 10% FCS. For all experiments, cells were plated at a density of 2 x 10^4 cells/cm^2 and grown to confluency for 10 days, with medium changes every 2–3 days.

Immunofluorescence

For fluorescence staining, HUVEC were plated (2 x 10^4 cells/cm^2) on Eppendorf Celllocate glass coverslips (Eppendorf, Hamburg, Germany) coated with 100 μg/ml collagen G (20°C, 24 h) and grown to confluency for 10 days. To label F-actin, cells were fixed for 10 min with 3.7% formaldehyde in PBS containing 1 mM Ca^2+ and 1 mM Mg^2+; permeabilized for 5 min in cold aceton (−20°C), and air dried. Coverslips were then incubated for 20 min with rhodamine phalloidin (1:200 in PBS; Molecular Probes, Eugene, OR) and mounted in Mowiol (Calbiochem) containing 0.2% p-phenylenediamine (Sigma) as anti-fading agent. For immunostaining of phosphorylated MLC, cells were fixed as above, permeabilized with 0.1% Triton X-100 for 5 min, and then blocked for 30 min with 10% normal goat serum. Coverslips were then incubated with polyclonal rabbit antibody against phosphorylated MLC, and the reaction was developed using 1:200 diluted goat anti-rabbit IgG (Dianova, Hamburg, Germany) and then were developed with luminol solution (Pierce) and exposed to Kodak X-OMAT films (Kodak, Rochester, NY).

Preparation of myosin-enriched cell fractions

Myosin-enriched fractions of HUVEC were prepared as described previously (3, 4). Briefly, HUVEC were plated on collagen-coated 100-mm diameter plates (Falcon, Becton Dickinson, Plymouth, UK.) and cultivated for 10 days. Monolayers were washed twice with ice-cold PBS (Sigma) and then homogenized with 0.5% Nonidet P40 for 1 h at 4°C. The homogenate was centrifuged at 20,000 g for 30 min at 4°C. The supernatant was diluted 1:2 in PBS, and then centrifuged for 10 min at 15,000 × g at 4°C. The pellet was then resuspended, and assayed for phosphatase activity using calcium and calmodulin-dependent protein phosphatase assay kit (Pierce, Rockford, IL) using BSA as standard. As tested by SDS-PAGE and Coomassie blue staining, protein preparations showed essentially only one band.

Microinjection

Microinjection was performed with a Compc Injet micromanipulator (Cell Biology Trading, Hamburg, Germany). Cells were plated and cultured on Celllocate coverslips (Eppendorf) as described above. V14RhoA was diluted with microinjection buffer (150 mM NaCl, 50 mM Tris, 5 mM MgCl_2, pH 7.5) and injected at a concentration of 1 μg/ml into the cytoplasm of HUVEC. Control injections with microinjection buffer did not produce any visible effect on cell morphology or cell organization. The microinjected volume was about 1–3 x 10^-15 liter/cell. Injected cells were identified by coinjecting rat IgG (5 mg/ml) followed by staining with FITC-conjugated goat anti-rat IgG (Dianova, Hamburg, Germany). For each experiment, at least 100 cells were injected and examined by fluorescence microscopy.

MLC phosphorylation

MLC phosphorylation was analyzed by SDS-PAGE and Western blotting. HUVEC were grown for 10 days in 6-well culture dishes and treated with LPS as indicated. Cells were lysed with boiling Laemmli sample buffer. Proteins were then electrophoretically transferred to nitrocellulose membranes and subjected to immunoblotting for 1 hour with anti-phospho-MLC Ab (1:1000 in Tris-buffered saline containing 0.3% Tween 20, washed three times, incubated for 1 h with anti-rabbit immunoglobulin, and then developed with luminal solution (Pierce) and exposed to Kodak X-OMAT films (Kodak, Rochester, NY).

Measurement of myosin-associated phosphatase activity

Phosphatase activity was determined in myosin-enriched cell fraction by measuring release of radioactivity from [32P]phosphorylase b. Phosphatase activity of cell homogenates was then quantified by measuring release of radioactivity from [32P]phosphorylase b.

Results

LPS induces MLC phosphorylation via Rho/Rho kinase in HUVEC

We previously reported that thrombin and P. multocida toxin can inhibit MLC phosphatase in HUVEC by a mechanism involving Rho and its target Rho kinase (3, 4). We proposed that in concert with Ca^2+/calmodulin-dependent MLC kinase, the lowered MLC phosphatase activity enhances MLC phosphorylation and endothelial cell contraction. Endothelial cell contraction is considered a hallmark of pathologically increased endothelial/vascular permeability (Fig. 1). Because it has been shown that LPS can stimulate endothelial cell contraction in a Ca^2+-independent manner, we asked whether LPS might signal via the Rho/Rho kinase pathway (8, 9). To test this possibility, we measured MLC phosphatase activity and MLC phosphorylation in HUVEC treated with LPS.
(100 ng/ml) for different time periods. As indicated in Fig. 2a, LPS stimulation markedly reduced MLC phosphatase activity in HUVEC, with a minimum of MLC phosphatase activity reached 1–2 h after LPS addition. MLC phosphatase down-regulation was followed by an increase in MLC phosphorylation that reached a maximum 3 h after LPS addition (Fig. 2b). In endothelial cells pretreated with the Rho inhibitor C3 transferase from C. botulinum (24 h, 5 μg/ml) or the Rho kinase inhibitor Y-27632 (30 min, 10 μM), no decrease in MLC phosphatase was detectable after LPS addition (Fig. 3b). In fact, MLC phosphatase almost remained at the elevated levels seen in the C3 transferase- or Y-27632-treated cells (Fig. 3a). Likely as a consequence of this in the C3 transferase- or Y-27632-pretreated cells, MLC phosphorylation stimulated by LPS was abolished (Fig. 4). Together these findings suggest that LPS inactivates MLC phosphatase via Rho/Rho kinase which produces enhanced MLC phosphorylation. Furthermore, elevation of MLC phosphatase by C3 transferase or Y-27632 treatment indicates that a basal Rho/Rho kinase activity keeps MLC phosphatase at an intermediate level in unstimulated endothelial cells. This might be advantageous because an intermediate level of MLC phosphatase can rapidly be altered in both directions: increased by Rho inhibition or decreased by Rho activation.

cAMP increases MLC phosphatase activity and blocks LPS-stimulated MLC phosphorylation

Given that elevation of intracellular cAMP can prevent endothelial cell contraction stimulated by LPS (8, 9), we asked whether the underlying mechanism might be inhibition of the Rho/Rho kinase pathway. To test this notion, endothelial cells were treated with combinations of cholera toxin (CTx) and isobutyl-methyl-xanthin (IBMX) or forskolin and dibutyryl cAMP to maximally elevate intracellular cAMP levels (17, 18). In such treated endothelial cells, MLC phosphatase increased to a similar degree as after C3 transferase or Y-27632 treatment (Fig. 3a). In addition, CTx/IBMX or forskolin/dibutyryl cAMP treatment inhibited the LPS-induced decrease in MLC phosphatase (Fig. 3b), and furthermore these substances blocked MLC phosphorylation stimulated by LPS in a manner similar to C3 transferase or Y-27632 (Fig. 4). These data strongly suggest that cAMP elevation inhibits the Rho/Rho kinase pathway, leading to increased MLC phosphatase activity and a blockage of MLC phosphorylation. To test whether the inhibitory cAMP effect on MLC phosphorylation is mediated by PKA, we treated cells with the highly selective and cell-permeable PKA-inhibiting peptide PKI-1422 (1 h, 40 nM) or with the PKA inhibitor H89 (1 h, 50 nM). If the inhibitory cAMP effect on LPS-induced MLC phosphorylation was mediated by PKA, it should be blockable by the PKA inhibitors. As indicated in Fig. 4, however, inhibition of LPS-induced MLC phosphorylation by cAMP could not be prevented by the PKA inhibitors, suggesting a PKA-independent mechanism.

cAMP elevation does not induce phosphorylation of Rho in HUVEC

We could confirm that recombinant RhoA is a good substrate for PKA in vitro whereas a serine 188 to alanine Rho mutant was not
phosphorylated (data not shown). To clarify whether phosphorylation of Rho takes place in intact HUVEC, we used two-dimensional gel electrophoresis because we failed to precipitate significant amounts of Rho with a variety of commercially available anti-RhoA Abs. Recombinant Rho, like many proteins, shows an acidic shift in two-dimensional gels upon phosphorylation due to the additional negative charge of the phosphate group (M. Aepfelbacher and M. Essler, unpublished observations). As demonstrated in Fig. 5a, RhoA in HUVEC appeared as two spots with pI values of 5.9 and 6.3, respectively, in two-dimensional gels. The nature of these two RhoA spots is not known, but in CTx/IBMX-treated cells none of the two RhoA spots shifted to a more acidic pH (Fig. 5b). To further test for phosphorylation of Rho, we labeled CTx/IBMX-treated HUVEC with inorganic [32 P]phosphate, separated phosphorylated proteins with two-dimensional gel electrophoresis, and blotted them onto a polyvinylidene difluoride membrane. The blot membrane was first subjected to autoradiography and then to anti-RhoA immunoblot. Although dozens of phosphorylated proteins were detected in CTx/IBMX-treated HUVEC, no phosphorylated protein comigrated with the two RhoA spots found in Western blot (Fig. 5, c and d). Altogether these data suggest that RhoA is not phosphorylated in HUVEC after elevation of cAMP.

cAMP-elevating agents do not inhibit downstream effectors of Rho

To determine more exactly at which level of the Rho/Rho kinase signaling pathway cAMP might act, we microinjected constitutively active V14Rho or inhibited MLC phosphatase with the drug calyculin A (15 min, 10 nM) in cells pretreated with CTx/IBMX. Thereafter, cells were stained with an Ab recognizing the phosphorylated forms of MLC only. Control cells showed almost no phosphorylated MLC (Fig. 6a). LPS treatment stimulated the appearance of phosphorylated myosin along stress fibers (Fig. 6b). Pretreatment of cells with CTx/IBMX prevented LPS-induced MLC phosphorylation and stress fiber formation (Fig. 6c). Micro-injection of V14Rho (Fig. 6d) caused cell contraction and MLC phosphorylation, mimicking the effect of LPS. However, this effect was not inhibited by pretreatment of cells with CTx/IBMX (Fig. 6e). Furthermore, treatment with calyculin A caused the appearance of phosphorylated MLC along stress fibers (Fig. 6f) but this could also not be inhibited by CTx/IBMX (Fig. 6g). Taken together, these data indicate that cAMP inhibits Rho/Rho kinase signaling by acting on an upstream regulator of Rho.
Inhibitors of transcription and translation prevent LPS-induced MLC phosphorylation

It is well documented that LPS enhances protein synthesis in endothelial cells by activation of transcription and translation. The newly formed proteins are partly secreted and can stimulate endothelial cells in a paracrine manner. Therefore, LPS-induced MLC phosphorylation could be due to activation of Rho/Rho kinase by secreted mediators like IL-6 (27). To test whether de novo synthesis of protein is required for LPS-induced MLC phosphorylation, we stimulated HUVEC with LPS (3 h, 100 ng/ml) in the presence of cycloheximide (10 \( \mu \)M) or actinomycin D (10 \( \mu \)M) to inhibit translation or transcription, respectively. As indicated by Fig. 7, both inhibitors blocked LPS-induced MLC phosphorylation, suggesting that MLC phosphorylation after LPS stimulation occurs secondary to protein synthesis.

Discussion

During septic shock, LPS induces endothelial cell contraction and formation of intercellular gaps which allows leaking of soluble and particulate blood components out of blood vessels. In this study, we describe a signal pathway by which LPS induces MLC phosphorylation, the key biochemical mechanism controlling cell contraction. Our data suggest that LPS activates the small GTP-binding protein Rho and its target Rho kinase, which inactivates MLC phosphatase leading to MLC phosphorylation.

LPS signaling in CD14-bearing cells such as monocytes and macrophages has been well characterized, while less is known about signal pathways stimulated by LPS in non-CD14-bearing cells such as human endothelial cells. It has been shown that activation of endothelial cells by LPS is dependent on soluble CD14 (sCD14) present in serum, which binds LPS to form a LPS-sCD14 complex (27). It is unclear which receptors are activated by the LPS-sCD14 complex and which second messengers these hypothetical receptors stimulate. Hence, the signal pathway by which LPS activates Rho in endothelial cells still remains to be investigated. It was suggested that heterotrimeric G protein-coupled receptors can activate Rho through p115 Rho GEF, a molecule that acts both as a GTPase-activating protein for G\( \alpha \) subunits and a nucleotide exchange factor for Rho (28). In fact, involvement of heterotrimeric G proteins in CD14-dependent LPS signaling was suggested recently (29). Another report suggested that the epidermal growth factor-receptor tyrosine kinase is an intermediary step between cell surface receptors and Rho activation (30). Interestingly, tyrosine
phosphorylation in endothelial cells could be that an increase in MLC phosphorylation for the time lag between LPS stimulation and MLC phosphorylation mechanism in HUVEC within minutes (31). One additional explanation for this observation is that an increase in MLC phosphorylation is not overcome by inhibition of PKA, suggesting that cAMP elevation inhibits basal Rho/Rho kinase activity in human endothelial cells. Because CAMP elevation does not interfere with MLC phosphorylation induced by constitutive active Rho or calcineurin A, we conclude that CAMP blocks an upstream RhoA regulator. CAMP elevation might, for example, interfere with GTP/GTP exchange on Rho as proposed to occur in mouse lymphocyte cell lines (32). Interestingly it was reported recently that CAMP regulates the activity of the rap guanine-nucleotide exchange factor Epac independently of PKA (33). Therefore, it could be speculated that CAMP utilizes another PKA-independent mechanism to inhibit the GDP/GTP exchange on Rho. It was shown that CAMP-dependent protein kinase A phosphorylates recombinant RhoA on serine 188 and that as a consequence Rho in intact cells might be inactivated (22). However, by two-dimensional gel analysis, we could not find any indication that RhoA is phosphorylated in endothelial cells. Furthermore, the effect of CAMP-elevating substances was not overcome by inhibition of PKA, suggesting that CAMP interferes with Rho signaling by a PKA-independent mechanism. It is well documented that the small GTP-binding protein Rac activates or inactivates Rho depending on the cell type (10, 34). Therefore, inactivation of Rho could also be caused by CAMP-mediated modulation of Rac activity. In this context, it was found that the CAMP-induced differentiation of melanoma cells is not only overcome by transfection of cells with constitutively active Rho, but also by transfection with constitutively active Rac (23). In any case, the direct target of CAMP in endothelial cells controlling Rho activity awaits further identification.

The Rho/Rho kinase pathway seems to be a crucial checkpoint for regulation of endothelial permeability in vitro and in vivo. Therefore, it could be envisioned that one therapeutic strategy during septic shock consists in the blockage of Rho/Rho kinase to prevent vascular leakage. CAMP-elevating drugs could be potentially useful for this purpose at present and the newly available Rho kinase inhibitors might be an option for the future.
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References