Regulation of endothelial cell myosin light chain kinase by Rho, cortactin, and p60SRC

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The role of Ser/Thr phosphorylation in the development of smooth muscle and nonmuscle contraction is well accepted (1, 5). Agonist-mediated increases in Ca²⁺/calmodulin (CaM) availability produces myosin light chain (MLC) kinase (MLCK)-mediated MLC phosphorylation on Ser³⁹⁷ and Thr⁷⁸³, resulting in actomyosin cross-bridge cycling and tension development. Garcia and colleagues (10, 13) have previously demonstrated that the physiological consequences of increased MLC phosphorylation in cultured vascular endothelium include endothelial cell (EC) contraction and the formation of paracellular junctional gaps. This loss of integrity of the semiselective EC barrier is now well known to facilitate the development of two cardinal features of tissue inflammation: leukocyte diapedesis and increases in vessel permeability (9, 10, 15, 18, 19, 28).

To better understand the regulation of EC contraction, we recently cloned a nonmuscle Ca²⁺/CaM-dependent EC MLCK isoform with a molecular mass (214 kDa) that is significantly greater than the conventional smooth muscle MLCK isoforms (130–160 kDa) (12, 48, 49). The activity of this key effector is regulated not only by Ca²⁺/CaM availability but by Ser/Thr phosphorylation of the enzyme as well (10, 12, 14, 49). Similar to the involvement of both EC MLCK and myosin-associated phosphatase activities in determining the extent of MLC phosphorylation in endothelium, the steady-state level of phosphotyrosine in most cellular proteins is a dynamic balance between the relative catalytic activities of intracellular protein tyrosine kinases and phosphatases. Although the precise role of tyrosine phosphorylation in the development of smooth muscle tension and force is incompletely understood (3, 6–8, 51), in prior work, Shi et al. (45) described the capacity of a tyrosine kinase inhibitor, genistein, to attenuate thrombin-induced tyrosine kinase activity, Ca²⁺ transients, MLC phosphorylation, and EC barrier dysfunction, suggesting a role for tyrosine kinase activity in EC barrier regulation. Unfortunately, neither the tyrosine kinase involved nor the relevant targets in this model were precisely identified, although indirect evidence suggested that a genistein-sensitive Src family tyrosine kinase may regulate EC MLCK activity (45).

The speculation that EC MLCK activity may be regulated by tyrosine phosphorylation was supported by companion studies that employed vanadate, an inhibitor of tyrosine phosphatases, to explore the contribution of tyrosine phosphorylation to EC barrier regulation (16). Vanadate treatment directly increased MLC phosphorylation in the absence of a rise in cytosolic Ca²⁺ and also directly perturbed EC barrier function (16). In the present study, we have extended these observations by examination of the participation of protein tyrosine phosphorylation in the activation of the EC actomyosin cytoskeleton. For these studies, we utilized the cell-permeable tyrosine kinase activator and tyrosine phosphatase inhibitor diperoxovanadate (DPV), which has been identified as the major peroxovanadium compound generated when equimolar amounts of H₂O₂ and sodium ortho- or metavanadate are mixed...
at a neutral pH (4, 17, 21, 37). Our results indicate that DPV evokes significant EC MLCK phosphorylation accumulation, increased EC MLCK activity, and EC contraction. These events appear to involve the activation of p60src, which exists in stable association with EC MLCK and the EC cytoskeleton and catalyzes the phosphorylation of EC MLCK as well as the p85 actin-binding protein cortactin. Furthermore, Rho GTPases appear to be major participants in determining the final level of DPV-induced MLC phosphorylation and contraction. Together, these data indicate the potential participation of Rho GTPases, p60src, and cortactin in the assembly of a functional MLCK complex and in EC contractile regulation.

METHODS

Reagents. Bovine EC cultures were maintained in DME (GIBCO BRL, Chagrin Falls, OH) supplemented with 20% (vol/vol) colostrum-free bovine serum (Irvine Scientific, Santa Ana, CA), 15 µg/ml of EC growth supplement (Collaborative Research, Bedford, MA), a 1% antibiotic-antimycotic solution (10,000 U/ml of penicillin, 10 µg/ml of streptomycin, and 25 µg/ml of amphotericin B; KC Biologicals, Lenexa, KS), and 0.1 mM nonessential amino acids (GIBCO BRL). Unless specified, reagents were obtained from Sigma (St. Louis, MO). Phosphate-buffered saline (PBS) and Hanks’ balanced salt solution without phenol red were purchased from GIBCO BRL (Grand Island, NY). Sodium orthovanadate (Na3VO4; vanadate) and hydrogen peroxide (H2O2) were obtained from Fisher Scientific (Fair Lawn, NJ). C3 exotoxin derived from Clostridium difficile was purchased from List (Campbell, CA). DPV was prepared as previously described (30). Polyacrylamide gels 4–15% ready-to-use gels were purchased from Bio-Rad (Hercules, CA).

Bovine and human pulmonary EC cultures. Bovine pulmonary arterial ECs were obtained frozen at 16 passages from American Type Culture Collection (Manassas, VA), utilized at passages 19–24, and cultured in complete medium (9). Human pulmonary microvascular endothelium was purchased from Clonetics (San Diego, CA) and utilized at passages 3–19 (48). The EC cultures were maintained at 37°C in a humidified atmosphere of 5% CO2-95% air and grew to contact-inhibited monolayers, with typical cobblestone morphology. Cells from each primary flask were detached with 0.05% trypsin, resuspended in fresh culture medium, and passaged into 60-mm dishes for immunoprecipitation studies, tyrosine protein phosphorylation determination, or MLC phosphorylation studies.

MLC phosphorylation in intact endothelium. EC monolayers grown in 60-mm tissue dishes were analyzed for MLC phosphorylation by urea PAGE as previously described by Garcia et al. (10), followed by Western immunoblotting with specific anti-MLC antibodies. The blot was scanned on a Bio-Rad densitometer, and the percent MLC phosphorylation was determined by dividing the total of the phosphorylated and nonphosphorylated areas. This method takes advantage of the fact that the mono- and diphasorylated forms of MLC migrate more rapidly than nonphosphorylated MLC and are independent of sample loading. Stoichiometry (in mol/mol) was calculated with the formula ([P1 + 2(P2)]/([U + P1 + P2]), where U is the percent unphosphorylated EC MLC, P1 is the percent monophosphorylated MLC, and P2 is the percent diphasorylated MLC. The diphasorylated MLC is multiplied by a factor of 2 to reflect the presence of two phosphate groups per light chain. The percentage of light chain phosphorylation was calculated by adding the densitometric values of each phosphorylation state for each isoform, i.e., unphosphorylated, monophosphorylated, and diphasorylated.

Western immunoblotting. Total protein extracts or immunoprecipitates solubilized in sample buffer were separated by 4–15% gradient SDS-PAGE (Bio-Rad), transferred to nitrocellulose (18 h at 30 V), and reacted with either antibodies to MLCK (D119) or anti-phosphotyrosine antibodies (Upstate Biotechnology) as previously described (14, 16, 49). Immunoreactive proteins were detected with an enhanced chemiluminescence detection system according to the manufacturer’s instructions (Amersham).

MLC immunoprecipitation. For immunoprecipitation under nondenaturing conditions, confluent ECs from 60-mm dishes were rinsed with medium 199 and two times with PBS, then lysed for 20 min on ice with 300 µl of Nonidet P-40 (NP-40) (NP-40) lysis buffer (1% NP-40, 20 mM MOPS, pH 7.0, 25 mM MgCl2, 10% glycerol, and 0.5 mM EDTA) containing protease inhibitors (40 µg/ml of aprotinin, 18 µg/ml of N-tosyl-l-phenylalalanine chloromethyl ketone, 6 µg/ml of Nα-p-tosyl-l-lysine chloromethyl ketone (TLCK), and 0.5 mM phenylmethylsulfonyl fluoride) (12, 16). The lysate was scraped and microcentrifuged for 5 min at 4°C, and the supernatant was used for immunoprecipitation. Each sample was diluted with 700 µl of washing buffer (0.1% NP-40, 50 mM MOPS, pH 7.0, 25 mM MgCl2, and 1 mM EDTA) and incubated overnight with 2 µl of anti-MLCK antibodies D119 at 4°C followed by incubation for 1 h at 4°C with 30 µl of 10% Pansorbin suspension (Formalin-hardened and heat-killed Cowan 1 strain Staphylococcus aureus cells purchased from Calbiochem (La Jolla, CA)). The immunoprecipitated complex was harvested by microcentrifugation, washed three times with washing buffer, and used for MLCK activity measurement (see Determination of MLCK activity) or resuspended in 200 µl of Laemmli sample buffer and heat treated at 110°C for 5 min. Immunoprecipitated proteins were separated from the Pansorbin beads by microcentrifugation for 1 min and subjected to Western immunoblotting analysis with specific antibodies to contractile proteins.

For immunoprecipitation under denaturing conditions, confluent EC monolayers in 60-mm tissue culture dishes were rinsed once with 2 ml of PBS, and scraped into 100 µl of SDS-denaturing stop solution (PBS, pH 7.4, 1 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 0.2 mM orthovanadate, 1% SDS, and 14 mM β-mercaptoethanol) (49, 50). The homogenate was prepared by passing the cell suspension several times through a 16-gauge needle. Homogenates were heat heated at 110°C for 5 min, diluted 1:10 with 900 µl of PBS, and incubated with 50 µl of 10% Pansorbin for 30 min at room temperature. Samples were clarified by microcentrifugation (5 min; Eppendorf), and the supernatants were incubated with 10 µl of anti-MLCK antibodies (60 min at room temperature or overnight at 4°C), then with 50 µl of 10% Pansorbin suspension for 60 min at room temperature. Immunocomplexes were pelleted by microcentrifugation for 5 min, washed three times with 1 ml of PBS, separated from Pansorbin by microcentrifugation, and subjected to SDS-electrophoresis (16, 27). After electrophoresis, the proteins were transferred to nitrocellulose membranes, and signals were detected by immunostaining with anti-phosphotyrosine antibodies.

Determination of MLCK activity. Kinase activity present in nondenaturing MLCK immunoprecipitates was determined as described by Garcia et al. (12), Gilbert-McClain et al. (16), and Heffetz et al. (17). Immunocomplexes were resuspended in 110 µl of 50 mM MOPS, pH 7.4, 10 mM magnesium acetate, 1 mg/ml of BSA, and 8 mM β-mercaptoethanol and preincu-
bated with and without the specific MLCK inhibitor KT-5926 (10 µM) for 15 min at 25°C. Kinase activity in MLCK immunoprecipitates was measured with baculovirus-expressed and His-Tag purified smooth muscle MLC (1 mg/ml) as a substrate in a buffer consisting of 50 mM MOPS, pH 7.4, 10 mM magnesium acetate, 1 mM of BSA, 1 µM CaM, 0.1 mM [γ-32P]-ATP (1 Ci/mmol), and 0.3 mM CaCl2 for 30 min at 25°C. The kinase reaction was stopped by pipetting aliquots onto Whatman P81 filters and immediately rinsing with ice-cold 10% TCA, 2% (wt/vol) sodium pyrophosphate, and 95% ethanol. Finally, filters were rinsed in ethyl ether, dried, and counted by liquid scintillation counting. Specific MLCK activity was defined in our assay conditions as the total kinase activity in nondenaturing MLCK immunoprecipitates sensitive to the specific MLCK inhibitor KT-5926 (12).

EC detergent fractionation. Confluent EC monolayers were partitioned into subcellular fractions with a protocol that is based on the detergent extractability of cell proteins under increasingly stringent conditions (39). Briefly, agonist- or DPV-stimulated cells grown to confluence in 100-mm dishes were rinsed with ice-cold PBS to remove nonadherent cells and then incubated for 10 min at 4°C in 1,500 µl of buffer A [0.01% digitonin, 10 mM PIPES, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 5 mM EDTA, and a protease inhibitor cocktail (0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 1 mM TLCK, and 25 µg/ml of leupeptin), pH 6.8]. The supernatant was harvested (cytosolic extract A), and adherent cells were then washed with 3 ml of buffer A and incubated for 20 min at 4°C in buffer B consisting of 0.5% (vol/vol) Triton X-100, 10 mM PIPES, pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 3 mM EDTA, and a protease inhibitor cocktail. The extract was harvested (Triton X-100-soluble extract B), and residual cellular material was next washed with 3 ml of buffer B and then scraped into 450 µl of buffer C (1% Tween 40, 0.5% deoxycholate, 10 mM PIPES, pH 7.4, 10 mM NaCl, 1 mM MgCl2, and a protease inhibitor cocktail). The cellular material was suspended by probe sonication on ice three times for 10 s and extracted with constant rotation at 4°C for 20 min. The insoluble elements were collected by centrifugation, and the supernatant was harvested (Triton X-100-insoluble extract C). The remaining insoluble cytoskeleton was washed with buffer C and suspended in 1% (wt/vol) SDS-5 mM Tris-HCl, pH 6.8, by probe sonication and rapidly boiled (5 min), divided into aliquots, and stored (cytoskeletal extract D).

Immunofluorescence. The fluorescent imaging of EC gap formation and F-actin organization was performed on EC monolayers grown to confluence on glass coverslips as previously described by Schaphorst et al. (44) and Verin et al. (49). After treatment, the cells were fixed by exchanging medium with 5% paraformaldehyde, 50 µM phosphate, 75 mM NaCl, and 25 mM Tris, pH 7, on ice for 10 min. The cells were then thoroughly rinsed with buffer containing 150 mM NaCl and 50 mM Tris, pH 7.6, and then permeabilized by a 3.5-min treatment with 0.2% Triton X-100 in the rinse buffer. The cells were again rinsed three times and incubated at room temperature for 1 h with 1% BSA in the rinse buffer and then with 1 U/ml of rhodamine phallolidin (Molecular Probes, Eugene, OR) to identify F-actin. Time-dependent changes in intracellular distribution of the actin cytoskeleton after a 5 µM DPV challenge were analyzed on a Zeiss axioplan fluorescence microscope. To study colocalization of actin and MLCK, the fixed permeabilized cells were exposed overnight at 4°C to a 1:50 dilution of anti-EC MLCK antibody (V-368) (12) in BSA buffer. This antibody was generated against the peptide GEERKRP present in the unique NH2-terminal part of EC MLCK (49). After being rinsed to remove unbound primary antibody, the cells were incubated for 1 h at room temperature with labeled secondary antibody (30 mg/ml; FITC-conjugated donkey anti-rabbit IgG, Jackson ImmunoResearch, West Grove, PA) and rhodamine phalloidin. The cells were examined with a ×60 oil objective with the Bio-Rad MRC 1024 confocal microscope and excitation with an Ar-Kr laser at 568-nm excitation and 598-nm emission for rhodamine and 488-nm excitation and 522-nm emission for FITC at a 3-µm aperture. Data were collected for 7–17 planar sections at 0.5-µm intervals by Bio-Rad LaserSharp acquisition software, processed by MetaMorph Imaging software (Universal, West Chester, PA), and printed on a thermal dye diffusion printer (Kodak, Rochester, NY). EC monolayers that were not exposed to primary antibody did not stain with the secondary antibody.

RESULTS

Effect of DPV on EC myosin phosphorylation and MLCK activity. To assess the linkage between DPV stimulation and the development of a contractile EC phenotype, initial experiments measured the capacity of DPV to increase the level of phosphorylated MLCKs. Confluent EC monolayers were stimulated for specified periods, and the stoichiometry of MLCK phosphorylation was determined by densitometric scanning of the immuno-oblots of MLCKs separated by urea gel electrophoresis. Figure 1A demonstrates that the combination of 10 µM H2O2 and 10 µM vanadate to generate DPV potently increases EC MLCK phosphorylation, whereas neither H2O2 alone (10 µM) nor vanadate alone (10 µM) altered the basal level of MLCK phosphorylation (stoichiometry of ~0.4 mol phosphate/mol MLCK). The maximal stoichiometric increase produced by DPV was ~1.8 mol phosphate/mol MLCK achieved with a 30-min stimulation with 10 µM DPV. Temporal analysis demonstrated that the increase in MLCK phosphorylation after DPV occurs as early as 5 min (Fig. 1B).

The level of MLCK phosphorylation represents the balance between EC MLCK and myosin-associated phosphatase activities (50). To explore the contribution of these components to DPV-stimulated MLCK phosphorylation, we pretreated EC monolayers with KT-5926, a well-described MLCK inhibitor (10, 12). Inhibition of EC MLCK with KT-5926 (4 µM) resulted in marked attenuation of DPV-mediated MLCK phosphorylation (Fig. 2), consistent with DPV-induced EC MLCK activation. We next immunoprecipitated EC MLCK from endothelium challenged with either vehicle, thrombin, or DPV and assessed in vitro kinase activity at specific times. Table 1 demonstrates a significant enhancement of MLCK activity after treatment with DPV, with maximal activity noted at 10 min, a time frame consistent with prior results by Gilbert-McClain et al. (16) that examined in vitro MLCK activity after vanadate stimulation.

Effect of DPV on EC MLCK translocation to the actin cytoskeleton. Having biochemically established that DPV activates EC MLCK and increases MLCK phosphorylation, we next examined the effect of DPV on the endothelial contractile apparatus and actomyosin cytoskeletal architecture by immunofluorescence confocal microscopy. Similar to thrombin and consistent with
the presence of a contractile phenotype, DPV increases F-actin-based stress-fiber formation and time-dependent reorganization of the F-actin-containing dense peripheral band normally present in a circumferential distribution in resting endothelium (Fig. 3) (9, 49). In unstimulated endothelium, immunoreactive MLCK was primarily cytosolic and did not colocalize with polymerized actin. Within 10 min after DPV challenge, however, there was a marked increase in stress-fiber assembly and polymerized actin and significant colocalization of EC MLCK with actin. This finding is consistent with EC MLCK translocation to the actin cytoskeleton as previously described in thrombin-challenged endothelium (49).

Table 1. Effect of DPV on MLCK activity in EC MLCK immunoprecipitates

<table>
<thead>
<tr>
<th>Agent</th>
<th>MLCK Phosphorylation Activity</th>
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<tbody>
<tr>
<td>Control (2–60 min)</td>
<td>100</td>
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<tr>
<td>Thrombin (2 min)</td>
<td>180 ± 29*</td>
</tr>
<tr>
<td>DPV 5 min</td>
<td>111 ± 13.5</td>
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<tr>
<td>10 min</td>
<td>130 ± 8.7*</td>
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<tr>
<td>20 min</td>
<td>121 ± 10.3</td>
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<tr>
<td>30 min</td>
<td>95 ± 9.4</td>
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<tr>
<td>60 min</td>
<td>129 ± 6*</td>
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Values are means ± SD in percent change from control value. Endothelial cell (EC) monolayers were treated with vehicle (medium 199), thrombin (100 nM), or diperoxovanadate (DPV; 5 µM) for indicated time periods. EC myosin light chain (MLC) kinase (MLCK) activity (1:10 dilution) in nondenaturing MLCK immunoprecipitates was determined with 5 µM MLC as a substrate (obtained with His-Tag by a baculovirus expression system) in presence of [γ-32P]ATP as described in METHODS. DPV significantly increased kinase activity in EC MLCK immunoprecipitates in a time-dependent manner, although extent of MLCK activity did not achieve that produced by thrombin. *Significant difference from control value, P < 0.05.

Effect of Rho GTPase inhibition on MLC phosphorylation in human and bovine endothelia. Having clearly demonstrated DPV-mediated activation of the 214-kDa
EC MLCK isoform, we next examined the possible contribution of MLC phosphatase activity to the DPV contractile response. Although Gilbert-McClain et al. (16) have previously failed to identify a significant effect of millimolar vanadate on total Ser/Thr phosphatase activity, the lack of complete inhibition of DPV-mediated MLC phosphorylation with the MLCK inhibitor KT-5926 (Fig. 2) suggested the possible contribution of Ser/Thr phosphatase inhibition to the integrated DPV response. To assess this possibility, we utilized the increasingly appreciated knowledge that MLC phosphorylation levels are potently modified by Rho GTPases via Rho kinase activity, stress-fiber formation, and MLC phosphorylation. Figure 4A demonstrates that, unlike fibroblasts, thrombin, but not lysophosphatidic acid, increases MLC phosphorylation in human lung microvascular ECs. We next pretreated human and bovine lung endothelia with C3 exotoxin, a specific Rho GTPase inhibitor derived from C. difficile. Figure 4 demonstrates that C3 exotoxin totally abolishes basal and both thrombin-mediated (Fig. 4B) and DPV-mediated (Fig. 4C) MLC phosphorylation. These results indicate that thrombin- and DPV-mediated Rho activation increases MLC phosphorylation and that in addition to MLC phosphatase inhibition, one key target for this regulation is EC MLCK. Together, these results indicate that DPV produces a contractile phenotype in cultured endothelium involving Rho-regulated, MLCK-driven MLC phosphorylation and subsequent actomyosin-mediated contraction.

Effect of DPV on MLCK phosphotyrosine accumulation. To more fully examine MLCK regulation, we next assessed the level of EC MLCK phosphotyrosine accumulation produced by DPV. DPV is known to dramatically increase the level of EC tyrosine-phosphorylated proteins via profound enhancement of tyrosine kinase activity and phosphatase inhibition (31). Figure 5 demonstrates the relatively rapid onset of phosphotyrosine immunoreactivity of EC MLCK, with substantial increases correlating with the extent of DPV-induced MLC phosphorylation (Fig. 1B). This DPV-mediated increase in both MLCK phosphotyrosine and MLCK activity, combined with prior studies by Gilbert-McClain et al. (16) and Shi et al. (45), suggests a strong mechanistic link between EC MLCK phosphotyrosine status and enzymatic activity.

Detection of stably associated proteins in MLCK immunoprecipitates. To further define the potential regulation of EC contraction evoked by DPV, we next immunoprecipitated EC MLCK under non-denaturing conditions and analyzed the proteins retrieved in stable immunoprecipitates. Detection of stably associated proteins in MLCK immunoprecipitates. To further define the potential regulation of EC contraction evoked by DPV, we next immunoprecipitated EC MLCK under non-denaturing conditions and analyzed the proteins retrieved in stable immunoprecipitates. Detection of stably associated proteins in MLCK immunoprecipitates. To further define the potential regulation of EC contraction evoked by DPV, we next immunoprecipitated EC MLCK under non-denaturing conditions and analyzed the proteins retrieved in stable immunoprecipitates. Detection of stably associated proteins in MLCK immunoprecipitates. To further define the potential regulation of EC contraction evoked by DPV, we next immunoprecipitated EC MLCK under non-denaturing conditions and analyzed the proteins retrieved in stable immunoprecipitates.
however, cortactin antisera readily detected the highly immunoreactive 85-kDa phosphoprotein in MLCK immunoprecipitates. Figure 6B demonstrates that, under basal conditions, cortactin immunoreactivity in nonde-natured EC MLCK immunoprecipitates is present as both the p80 and p85 isoforms. After DPV stimulation, there is a significant increase in the level of the p85 cortactin isoform present within MLCK immunoprecipitates in close association with enhanced tyrosine phosphorylation of cortactin. Both c-Src and cortactin immunoprecipitates under basal conditions yielded both p80 and p85 cortactin isoforms, with an increase in the p85 cortactin isoform after DPV stimulation (Fig. 6B). Figure 7 demonstrates the rapid translocation of p80 and p85 cortactin from the cytosol to the EC cytoskeleton after DPV, with dramatic time-dependent increases in cortactin phosphotyrosine accumulation beginning at 5 min. Together, these data are consistent with the stable association of both p60<sup>Src</sup> and p80/85 cortactin with EC MLCK and suggests a potential mechanism for regulation of EC MLCK by tyrosine phosphorylation catalyzed by p60<sup>Src</sup>.

**DISCUSSION**

This study was undertaken to explore the poorly understood relationship between tyrosine kinase or phosphatase activities and nonmuscle contraction. The results reported are highly consistent with a major role of tyrosine phosphorylation in the regulation of the EC contractile apparatus, a process that appears to be regulated by Rho GTPTases and potentially driven by p60<sup>Src</sup>-regulated activity of a novel high-molecular-mass EC MLCK isoform. Prior structure or function studies (10, 14) indicated that activity of the Ca<sup>2+</sup>/CaM-dependent EC MLCK isoform is not solely regulated by Ca<sup>2+</sup>/CaM availability. Additional regulatory mechanisms have been postulated, including the subcellular localization of the EC MLCK isoform and posttranslational modifications such as Ser/Thr phosphorylation (12, 49). Although studies of the nonmuscle MLCK isoform are limited, the vast majority of studies dealing with phosphorylation of the smooth muscle MLCK isoform have demonstrated an inhibitory effect of this modification (46). In cultured endothelium, increases in cAMP-dependent protein kinase A activity or Ser/Thr phosphatase inhibition results in EC MLCK hyperphos-

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**Fig. 4.** Effect of C<sub>3</sub> exotoxin on Thr- and DPV-stimulated MLC phosphorylation. A: confluent bovine pulmonary arterial endothelial cells (BPAEC) and human lung microvascular ECs (HLMVEC) were challenged with Thr for 2 min or lysophosphatidic acid (LPA) for 15 min and assessed for level of phosphorylated MLC by urea gel electrophoresis. Unlike bovine endothelium, human endothelium exhibits both smooth muscle and nonmuscle isoforms, and this gives rise to 6 MLC species. Thr, but not LPA, a known G protein activator in fibroblasts (32), produced a sharp increase in phosphorylated MLC. B and C: bovine pulmonary arterial endothelium was briefly pretreated with lipofectamine (10 µM) and C<sub>3</sub> exotoxin (C3 Exo or C3; 5 µg/ml) followed by replacement with lipofectamine-free medium containing C3 Exo or vehicle (control (Cont)). Thr (2 min; B) or DPV (15 min; C) was added, and MLC phosphorylation profiles were analyzed. –, Absence; +, presence. These results indicate total inhibition of MLC phosphorylation (basal and after agonists) by Rho GTPase inhibition. Similar results were demonstrated in human microvascular endothelium after Thr and DPV (data not shown).

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**Fig. 5.** Effect of DPV on EC MLCK tyrosine phosphorylation. Shown is a representative experiment (n = 4) where EC monolayers were treated with DPV (5 µM) at specified time periods and MLCK immunoprecipitates were prepared under denaturing conditions from DPV-stimulated (+) and control (−) cells. Resulting samples were subjected to a 4–15% gradient SDS-PAGE, electrotransferred to nitrocellulose membranes, and reacted with anti-phosphotyrosine antibodies followed by enhanced chemiluminescence. Immunoprecipitation was performed with anti-MLCK D119 antibodies. No. on left, molecular-mass marker. DPV treatment increased total MLCK phosphotyrosine protein content in MLCK immunoprecipitates.
Ser/Thr phosphorylation in association with decreased MLCK activity in MLCK immunoprecipitates (12, 49). Analysis of the EC MLCK amino acid sequence reveals a number of consensus sites for Ser/Thr phosphorylation catalyzed by a variety of enzymes including protein kinase C, cAMP-dependent protein kinase A, cGMP-dependent protein kinase, casein kinase II, and Ca²⁺/CaM-dependent protein kinase II (11, 49). Although the Ser/Thr phosphatase associated with EC MLCK is, by implication, an essential regulator of MLCK activity, the exact nature of this key enzyme remains incompletely understood. Importantly, both prior reports by Gilbert-McClain et al. (16) and Shi et al. (45) and current data indicate that phosphorylation of EC MLCK on tyrosine residues, in contrast to the inhibitory effects of Ser/Thr phosphorylation, may participate in EC MLCK regulation in a stimulatory capacity.

DPV is a particularly well-suited tool with which to study the signaling cascades that regulate the EC contractile apparatus through tyrosine phosphorylation. Via its potent stimulatory effect on tyrosine kinases and robust inhibition of tyrosine phosphatases, DPV induces association of multiple tyrosine-containing proteins with Src homology (SH2 and SH3) domains (24) such as phospholipase C-γ, Src family tyrosine kinases, and potentially important adaptor proteins such as p59 Shc (22, 34, 38, 42). Careful analysis of the 1,914-amino acid sequence of EC MLCK reveals two SH2 binding sites (Tyr59 and Tyr464), two potential SH3 domains (amino acid residues 314–318 and 373–379), and consensus sites for tyrosine kinase phosphorylation catalyzed by the Src family of kinases (Tyr485, Tyr1449, and Tyr1575). Each SH2 and SH3 bind-

Fig. 6. DPV-mediated association of EC MLCK with p60src and cortactin. A: EC MLCK immunoprecipitates (I/P) from bovine EC extracts were prepared under nondenaturing conditions as described in METHODS and subjected to a 4–15% gradient SDS-PAGE. This was followed by Western immunoblotting with anti-phosphotyrosine (PTyr) antibodies, demonstrating a large number of phosphoproteins under these nondenaturing conditions. Membranes were then stripped and reprobed with specific smooth muscle MLCK D119 antibodies, anti-p60src, or anti-cortactin antisera. Arrows, positions of corresponding contractile proteins and rabbit IgG. These results indicate presence of an Src kinase (p60src) and several Src kinase substrates, including cortactin, in stable association with EC MLCK. B: immunoprecipitates prepared by utilizing MLCK antisera, c-Src antisera, or cortactin antisera under nondenaturing conditions were rebotted with anti-p80/85 cortactin antisera. An increase in immunoreactive cortactin after DPV (15 min) is observed in each experiment, primarily in p85 species. Nos. on left, molecular mass.

Fig. 7. Effect of DPV on cortactin tyrosine phosphorylation. ECs grown to confluence were challenged with 5 µM DPV for up to 30 min (30⁵⁸) and then partitioned by serial detergent fractionation as described in METHODS. Proteins present in digitonin-extracted fraction (cytosol) and SDS-extracted fraction (cytoskeleton) for each condition were separated on 8% acrylamide gels by SDS-PAGE and electrophoretically transferred to nitrocellulose followed by Western immunoblotting. Each lane represents 10 µg of total protein. These data indicate rapid translocation of immunoreactive cortactin (CA) from cytosol to cytoskeleton in concert with alterations in phosphotyrosine immunoreactivity. Similarly, an increase in cytoskeleton-associated actin is noted beginning 5 min (5⁵⁸) after DPV and persists throughout period tested. Nos. on left, molecular mass.
REGULATION OF EC MLCK BY Rho, Src, and Cortactin

Although our data provide a strong linkage between MLCK activity and the phosphotyrosine status of the kinase, we cannot exclude the possibility that additional DPV-mediated effects, aside from direct tyrosine phosphorylation of MLCK, may be contributing to kinase activation. For example, the increase in MLCK activities elicited by DPV may be related to DPV-mediated increases in EC cytosolic Ca\(^{2+}\), an essential cofactor in enhanced EC MLCK activation (10). However, even submillimolar concentrations of H\(_2\)O\(_2\) alone do not increase EC MLC phosphorylation (Fig. 1A), and previously published findings by Gilbert-McClain et al. (16) and Natarajan et al. (31) indicated that DPV and 100 \(\mu\)M H\(_2\)O\(_2\) only weakly increase cytosolic Ca\(^{2+}\) concentration, whereas millimolar concentrations of vanadate fail to alter Ca\(^{2+}\) in confluent endothelium. Furthermore, recent studies (10, 14) have unequivocally demonstrated that rises in cytosolic Ca\(^{2+}\), including those elicited by Ca\(^{2+}\) ionophores such as A-23187 and ionomycin, although necessary, are insufficient to activate the EC MLCK isoform. Ionomycin not only fails to activate MLCK but produces rapid MLC dephosphorylation via activation of the type 2B Ser/Thr phosphatase known as calcineurin (47, 50). Because extensive homology exists between the 130- to 160-kDa smooth MLCK isoform and the 214-kDa EC MLCK isoform in the CaM-binding region (residues 1749-1759), differences in CaM affinity are unlikely to explain the lack of enzymatic activation by Ca\(^{2+}\) alone (12, 48). It is much more likely that additional regulatory elements contained in the unique NH\(_2\)-terminal region (residues 1–922), not shared by smooth muscle MLCK isoforms, participate in EC MLCK regulation. Our data now indicate the strong possibility that MLCK tyrosine phosphorylation mediated by p60\(^{src}\) is an important event in the regulation of MLCK enzymatic activity and activation of the EC contractile apparatus.

One of the primary functions of the endothelium is to change its shape in response to agonists, a response that requires an extensive and rapid reorganization of the EC cytoskeleton. The Rho GTPase members of the Ras superfamily are clearly involved in the regulation of the nonmuscle cytoskeleton and stress-fiber formation (2, 32, 40). Although Gilbert-McClain et al. (16) have previously failed to identify a significant effect of millimolar vanadate on total Ser/Thr phosphatase activity, the lack of total inhibition of DPV-mediated MLC phosphorylation with KT-5926 suggested the possible contribution of Ser/Thr phosphatase inhibition to the full integrated DPV-induced MLC phosphorylation response similar to what Verin et al. (47, 50) have observed after thrombin stimulation. MLC phosphorylation in smooth muscle and nonmuscle tissues is potentially modified by Rho GTPases via Rho kinase-mediated phosphorylation of the regulatory subunit of MLC phosphatase (26). Our experiments utilizing C\(_3\) exotoxin, a specific Rho GTPase inhibitor, indicate that thrombin and DPV mediate significant Rho activation in human lung microvascular and bovine pulmonary endothelia, with C\(_3\) exotoxin totally abolishing basal thrombin- and DPV-mediated MLC phosphorylation. The mechanism of DPV-stimulated Rho activation is unknown; however, these results are entirely plausible because Rho activation has been shown to involve tyrosine kinase activities (32, 40). Therefore, the highly significant inhibition of DPV-mediated MLC phosphorylation by KT-5926, an MLCK inhibitor with relative specificity, in conjunction with the complete abolishment of MLC phosphorylation by C\(_3\) exotoxin, argues for an important role of the EC MLCK isoform in the DPV-mediated MLC response. Together, these results indicate that DPV-mediated Rho activation increases MLC phosphorylation and that key targets for this regulation appear to include both MLC phosphatase as well as the EC MLCK isoform. The novel mechanisms by which Rho may alter MLCK activities are under current investigation.

Our data examining proteins that are stably associated with EC MLCK indicate that p80/85 cortactin, a potent F-actin-binding protein associated with the nonmuscle cytoskeleton, may participate in a signaling cascade that enables the cytoskeleton to reorganize and the endothelium to remodel rapidly. Cortactin promotes sedimentation of F-actin at centrifugation forces under which F-actin is otherwise not able to be precipitated. However, cortactin is an in vitro substrate for p60\(^{src}\) (52, 53) and cortactin phosphorylation by Src abolishes F-actin bundling properties (20). Tyrosine phosphorylation of cortactin is immediately enhanced before thrombin-induced platelet aggregation and subsequent cortactin translocation to the cytoskeleton (41). Our data indicate that DPV induces cortactin phosphotyrosine phosphorylation (with a shift to the p85 isoform) and cortactin translocation to the endothelial cytoskeleton in a stable association with EC MLCK. The observations that 1) DPV significantly increases protein tyrosine phosphorylation of cytoskeletal elements, including both p80/85 cortactin and EC MLCK; 2) cortactin is present in c-Src immunoprecipitates; and 3) a stable association exists between p60\(^{src}\) cortactin and MLCK together implicate p60\(^{src}\) as the tyrosine kinase most likely responsible for phosphorylation of EC MLCK and cortactin. Recent reports (30, 33, 43) in cells lacking the c-src gene or after overexpression of c-Csk, a negative regulator of p60\(^{src}\), provide further
compelling evidence that cortactin is an intrinsic substrate for p60^Src. The deduced amino acid sequence of cortactin contains an SH3 motif at the COOH terminus and a NH2-terminal domain composed of five (p80) or six (p85) internal tandem repeats that represent sites of actin binding (20). The presence of an SH3 domain within the cortactin structure could support its function as a scaffolding protein with a number of Src family kinases, adaptor proteins, and specific cytoskeletal proteins as well as with signaling molecules (23). Both structural and subcellular localization data seem to support the concept that cortactin potentially participates in the transduction of signals from the cell surface to the cytoskeleton. In addition, the large number of known cytoskeletal targets for p60^Src, together with our observation of DPV-induced tyrosine phosphorylation of several proteins in the endothelial cytoskeleton, appears to be consistent with this notion.

In summary, we have used the cell-permeable oxydant and potent tyrosine kinase activator or phosphatase inhibitor DPV to study whether tyrosine phosphorylation is intimately involved in activation of the endothelial actomyosin cytoskeleton. Our results indicate that DPV activates EC MLCK in a Rho GTPase-dependent manner, stimulates EC MLCK phosphorytrosine accumulation, and promotes the stable association of p80/85 cortactin and p60^Src with EC MLCK and the actin cytoskeleton. Both cortactin and p60^Src can now be included as potentially key components involved in the establishment of a functional MLCK enzymatic complex known to include MLC and myosin heavy chain, CaM, and actin (49). We speculate that tyrosine phosphorylation of cortactin and MLCK by p60^Src within a growing MLCK complex of proteins may be key steps in a cascade of events leading to cytoskeleton changes, disassembly of lung EC adherens junctions, and, ultimately, leukocyte infiltration and edema formation during lung inflammation.

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