MARCKS protein mediates hydrogen peroxide regulation of endothelial permeability

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Impairment of endothelial barrier function is implicated in many vascular and inflammatory disorders. One prevalent mechanism of endothelial dysfunction is an increase in reactive oxygen species under oxidative stress. Previous reports have demonstrated that hydrogen peroxide (H_2O_2), a highly stable reactive oxygen species that modulates physiological signaling pathways, also enhances endothelial permeability, but the mechanism of this effect is unknown. Here, we identify the actin-binding protein myristoylated alanine-rich C-kinase substrate (MARCKS) as a key mediator of the H_2O_2-induced permeability change in bovine aortic endothelial cells. MARCKS knockdown and H_2O_2 treatment alter the architecture of the actin cytoskeleton in endothelial cells, and H_2O_2 induces the phosphorylation and translocation of MARCKS from the cell membrane to the cytosol. Using pharmacological inhibitors and small interference RNA constructs directed against specific proteins, we uncover a signaling cascade from Rac1 to Abl1, phospholipase Cγ1, and PKCδ that is triggered by H_2O_2 and leads to MARCKS phosphorylation. Our findings establish a distinct role for MARCKS in the regulation of H_2O_2-induced permeability change in endothelial cells, and suggest potential new therapeutic targets for the treatment of disorders involving oxidative stress and altered endothelial permeability.

The vascular endothelium forms the inner lining of blood vessels and functions as a selective barrier for transport of macromolecules and circulating cells (1, 2). Endothelial cells maintain barrier function by sustaining the integrity of the vessel wall. Multiple mediators affect the barrier function of endothelial cells. One family of mediators consists of the reactive oxygen species (ROS) (3) hydrogen peroxide (H_2O_2) and superoxide anion. ROS have physiological roles at lower concentrations, but higher concentrations of ROS induce oxidative stress and contribute to the pathophysiology of vascular diseases (4, 5), in part by causing endothelial barrier dysfunction (6, 7).

H_2O_2 is a cell-permeant and stable ROS generated mainly by the dismutation of superoxide anion by superoxide dismutases. H_2O_2 modulates diverse physiological processes in endothelial cells, including cytoskeletal reorganization, as well as vascular remodeling and vasorelaxation (8–10). The molecular mechanisms by which H_2O_2 affects these endothelial cell functions are incompletely understood. Treatment of endothelial cells with H_2O_2 modulates diverse signaling pathways (11–13), but the pathways that regulate actin polymerization and cytoskeletal reorganization have not been elucidated.

The MARCKS (myristoylated alanine-rich C-kinase substrate) protein is expressed in neuronal tissues and endothelial cells, where the protein has been implicated in the regulation of cell attachment and affects the directed migration of endothelial cells (14–16). MARCKS binds to actin and to calcium/calmodulin, and interacts with membrane phospholipids (17, 18). The binding of membrane phospholipids by MARCKS enables MARCKS association with the cell membrane. PKC-mediated MARCKS phosphorylation causes MARCKS to dissociate from the membrane and inhibits its ability to cross-link F-actin (18, 19). MARCKS's function as both a PKC substrate and an actin-regulating protein suggest that MARCKS could be a mediator in the regulation of H_2O_2-stimulated cytoskeletal reorganization in endothelial cells.

Here we study the regulation of endothelial permeability and cytoskeletal organization by H_2O_2. MARCKS is identified as a mediator of the H_2O_2-induced endothelial permeability change. MARCKS and H_2O_2 also affect the architecture of the actin cytoskeleton in endothelial cells. MARCKS is phosphorylated in response to H_2O_2 in a dose- and time-dependent manner, and H_2O_2 induces MARCKS translocation from the cell membrane to the cytosol. Experiments using small interference-(siRNA) targeting constructs identify an H_2O_2-induced Rac1/Abl1/phospholipase C (PLC)γ1/PKCδ signaling cascade that leads to MARCKS phosphorylation in endothelial cells. Our studies establish a unique role for MARCKS in regulating endothelial permeability.

Results

MARCKS Is an Important Mediator of the H_2O_2-Induced Endothelial Permeability Change and Actin Cytoskeleton Reorganization. We explored the role of MARCKS in the H_2O_2-induced increase in endothelial permeability, using a siRNA construct to specifically knock down MARCKS expression in bovine aortic endothelial cells (BAECs) (20). BAECs were chosen for these studies because of their phenotypic stability in cell culture and their well-characterized signaling pathways. We measured the H_2O_2-induced endothelial permeability change using a FITC-Dextran assay. BAECs transfected with MARCKS siRNA showed reduced MARCKS expression (Fig. S1). In the absence of H_2O_2, siRNA-mediated knockdown of MARCKS had no effect on endothelial permeability (Fig. 1*A*). In response to H_2O_2, cells transfected with control siRNA showed a significant increase in endothelial permeability; cells transfected with MARCKS siRNA did not show an increase in endothelial permeability for up to 3 h after H_2O_2 treatment. Four hours after H_2O_2 treatment, BAECs transfected with MARCKS siRNA did show a significant increase in endothelial permeability compared with untreated cells ($n = 4$, $P < 0.05$). However, the increase in permeability in H_2O_2-treated cells transfected with MARCKS siRNA was significantly less than in H_2O_2-treated cells transfected with control siRNA ($n = 4$, $P < 0.05$) (Fig. 1*A*). H_2O_2 induced a dose-dependent increase in endothelial permeability that was suppressed by siRNA-mediated MARCKS knockdown (Fig. 1*B*), with no change in endothelial cell viability. These results suggested that MARCKS is a mediator of the H_2O_2-induced endothelial permeability change, but does not modulate changes in basal permeability (Fig. 1).

We next examined the role of MARCKS in H_2O_2-mediated cytoskeletal reorganization. BAECs transfected with control or MARCKS siRNA were stained with Alexa Fluor-488 phalloidin to image the actin cytoskeleton. Endothelial cells contain stress fibers that form when bundles of actin filaments extend from the

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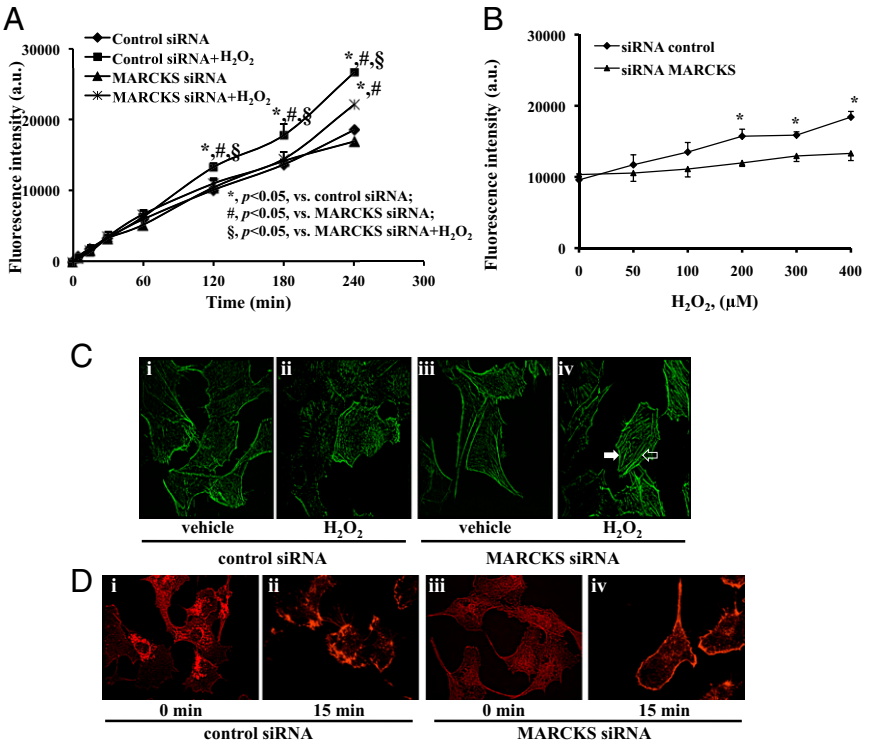

Fig. 1. MARCKS regulates endothelial permeability and cytoskeleton organization. (*A*) BAECs were transfected with control siRNA (diamond and square) or MARCKS siRNA (triangle and star), and endothelial permeability was assessed using the FITC-dextran assay. Cells were treated with vehicle (diamond and triangle) or with 200 μM H_2O_2 (square and star). The plot shows the fluorescence intensity of FITC-dextran ($n = 4$). $^*P < 0.05$ vs. control siRNA; $^{\#}P < 0.05$ vs. MARCKS siRNA; $^{\S}P < 0.05$ vs. MARCKS siRNA+H_2O_2. (*B*) Cells transfected with control siRNA or with MARCKS siRNA were treated with vehicle or with H_2O_2 at varying concentrations as indicated and analyzed for permeability. The plot shows the fluorescence intensity of FITC-dextran ($n = 3$). $^*P < 0.05$ vs. control siRNA. (*C*) BAECs were fixed, stained with Alexa Fluor-488 phalloidin, and imaged using a 60× objective. Cells transfected with control siRNA were treated with vehicle (i) or with 200 μM H_2O_2 (ii) for 30 min; cells transfected with MARCKS siRNA were treated with vehicle (iii) or with 200 μM H_2O_2 (iv) for 30 min. The open arrow demonstrates stress fibers; the solid arrow shows cortical actin. (*D*) BAECs were fixed and stained with Alexa Fluor-568 phalloidin to label actin. Cells transfected with control siRNA were treated with vehicle (i) or with 5 μM cytochalasin D (ii) for 15 min; cells transfected with MARCKS siRNA were treated with vehicle (iii) or with 5 μM cytochalasin D (iv) for 15 min.

cell surface through the cytosol (19). Actin stress fibers play an important role in regulating cellular adhesion, morphology, and permeability. Both siRNA-mediated knockdown of MARCKS and treatment with H_2O_2 increased the presence of actin stress fibers in BAECs (Fig. 1*C*). Compared with the actin stress fibers in BAECs transfected with MARCKS siRNA or treated with H_2O_2, the actin stress fibers in H_2O_2-treated BAECs transfected with MARCKS siRNA were more pronounced. In contrast, cortical actin, which is found just beneath the plasma membrane and is a key determinant of cell shape, was less evident in H_2O_2-treated BAECs transfected with control siRNA, and was more prominent in both untreated and H_2O_2-treated BAECs transfected with MARCKS siRNA (Fig. 1*C*). Treatment with cytochalasin D, which inhibits actin polymerization and promotes its depolymerization (21), disrupted the actin cytoskeleton in BAECs transfected with control siRNA (Fig. 1*D*). MARCKS knockdown mitigated the effect of cytochalasin D and preserved the cortical actin, enabling the cell to better retain its shape (Fig. 1*D*). These findings indicated the importance of MARCKS not only in the basal regulation of the actin cytoskeleton, but also in H_2O_2-modulated cytoskeletal reorganization.

H_2O_2 Induces MARCKS Phosphorylation and Translocation in Endothelial Cells. MARCKS phosphorylation and localization affect its binding partners and function (13, 18). We next characterized the effects of H_2O_2 on MARCKS phosphorylation and localization. Endothelial cells were treated with varying concentrations of H_2O_2, and cell lysates were analyzed in immunoblots probed with phospho-MARCKS and total MARCKS antibodies. H_2O_2 treatment increased MARCKS phosphorylation in a dose-dependent manner, without affecting total MARCKS abundance (Fig. 2*A*). MARCKS was phosphorylated within 15 min after addition of H_2O_2, and this signal was sustained for at least 60 min (Fig. 2*B*).

Phosphorylated MARCKS disassociates from the plasma membrane and translocates to the cytosol (13). We transfected BAECs with a plasmid encoding a MARCKS-GFP fusion protein and used laser confocal microscopy to image the fluorescent MARCKS construct. In untreated endothelial cells, almost all of the MARCKS-GFP protein was detected at the plasma membrane (Fig. 2*C*). Within 10 min after addition of H_2O_2, we observed a marked decrease in membrane-associated MARCKS-GFP accompanied by an increase in cytosolic MARCKS-GFP; the enhancement in cytosolic fluorescence was sustained for at least 60 min. Taken together, our results showed that H_2O_2 promotes MARCKS phosphorylation and induces MARCKS translocation from the plasma membrane to the cytosol.

PKCδ Is Required for H_2O_2-Induced MARCKS Phosphorylation. The PKC family can catalyze the phosphorylation of MARCKS (17). We used a PKC inhibitor, Gö6983, to study the role of PKC in H_2O_2-modulated MARCKS phosphorylation in BAECs. Treatment with Gö6983 attenuated basal MARCKS phosphorylation and blocked the H_2O_2-induced increase in MARCKS phosphorylation (Fig. 3*A*). Many different PKC isoforms have been identified in mammals (22, 23); of these, the α, δ, and ε isoforms are dominantly expressed in BAECs (24). We designed and validated siRNAs to knock down these PKC isoforms (Fig. 3*B* and Fig. S2). siRNA-mediated knockdown of PKCδ abrogated MARCKS phosphorylation and suppressed MARCKS phosphorylation in response to H_2O_2 (Fig. 3*B*). In contrast, siRNA-mediated knockdown of either PKCα or PKCε had no effect on MARCKS phosphorylation (Fig. S2). These results established PKCδ as the critical isoform involved in MARCKS phosphorylation in response to H_2O_2.

We next examined the role of PKCδ in endothelial permeability. As previously reported (25), siRNA-mediated knockdown of PKCδ increased endothelial permeability (Fig. 3*C*). However, the H_2O_2-induced increase in endothelial permeability was absent in BAECs following siRNA-mediated PKCδ knockdown, implicating PKCδ-mediated MARCKS phosphorylation in the H_2O_2-enhanced endothelial permeability change. Phosphorylation of membrane-associated MARCKS requires the recruitment of the upstream kinases to the cell membrane. Confocal imaging using a PKCδ isoform-specific antibody showed that PKCδ was located mainly in the cytoplasmic region in the basal state (Fig. S2*C*). Addition of H_2O_2 induced PKCδ translocation to the plasma membrane (Fig. S2*C*, *Center* and *Right*). The recruitment

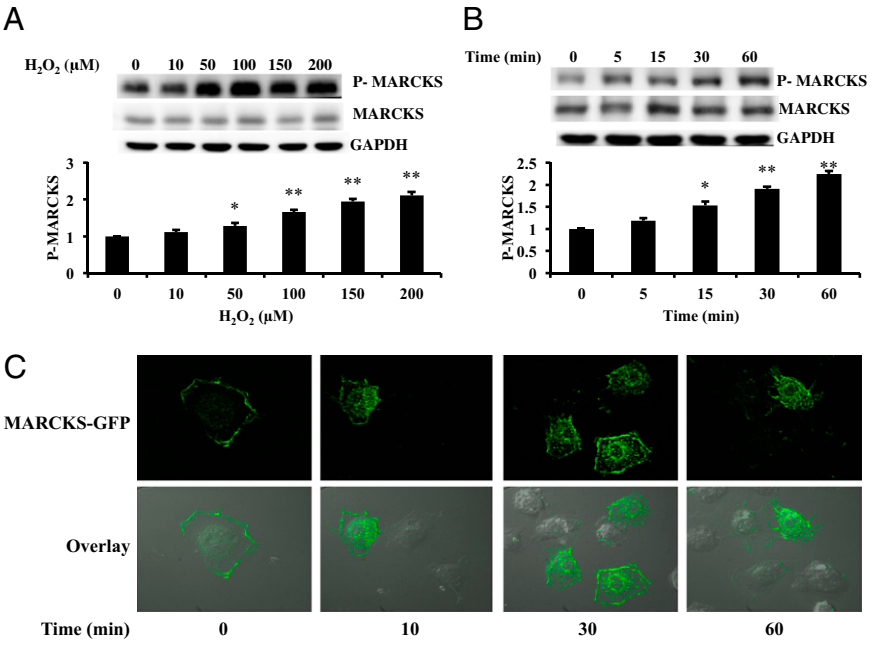

Fig. 2. H_2O_2 induces dose-dependent and time-dependent MARCKS phosphorylation and translocation. (*A*) BAECs were treated with various concentrations of H_2O_2 for 30 min and analyzed in immunoblots. (*Upper*) A representative immunoblot; (*Lower*) pooled data from five independent experiments. (*B*) BAECs were treated with 200 μM H_2O_2 for various times and analyzed in immunoblots. (*Upper*) A representative immunoblot; (*Lower*) pooled data from eight independent experiments. **P* < 0.05; ***P* < 0.01. (*C*) BAECs were transfected with MARCKS-GFP, treated with 200 μM H_2O_2 for the indicated times, and imaged using a 60× objective. (*Upper*) MARCKS-GFP fluorescence; (*Lower*) overlay images of MARCKS-GFP (green) with differential interference contrast images of the same cells (grayscale).

of PKCδ to the plasma membrane in response to H_2O_2 could facilitate MARCKS phosphorylation.

PLCγ1 Regulates H_2O_2-Induced MARCKS Phosphorylation. PKCδ is an atypical PKC isoform, the activation of which is independent of calcium but remains dependent on diacylglycerol (26). Because a rapid increase in diacylglycerol results mainly from PLC activity, we used the PLC inhibitor U73122 to assess the role of PLC in MARCKS phosphorylation. As shown in Fig. 4*A*, treatment with U73122 completely suppressed MARCKS phosphorylation. Because PLC is also activated by calmodulin-dependent protein kinase II (CaMKII) (27), which in turn can be activated by H_2O_2, we studied the specific CaMKII inhibitor KN93. We found that KN93 has no effect on MARCKS phosphorylation (Fig. S3), suggesting that H_2O_2 induces MARCKS phosphorylation independent of CaMKII activation.

PLC has multiple isoforms, and BAECs express the β1, β2, γ1, and δ1 isoforms (28); of these, the PLCγ1 isoform is activated by both receptor and nonreceptor tyrosine kinases (28). Because H_2O_2 has been implicated in activating several protein tyrosine kinases (29), we hypothesized that PLCγ1 could play a role in H_2O_2-induced MARCKS phosphorylation. siRNA-mediated knockdown of PLCγ1 suppressed H_2O_2-induced MARCKS phosphorylation but had no effect on basal phosphorylation (Fig. 4*B*). Furthermore, PKCδ phosphorylation at Thr505, an indicator of PKCδ activation, was reduced in BAECs transfected with PLCγ1 siRNA and stimulated with H_2O_2 (Fig. 4*B*). These results suggested that PLCγ1 activation is upstream of PKCδ-mediated MARCKS phosphorylation by H_2O_2.

Activation of Abl1 Is Upstream of PLCγ1/ PKCδ-Mediated MARCKS Phosphorylation by H_2O_2. The involvement of PLCγ1 in H_2O_2-induced MARCKS phosphorylation led us to investigate protein tyrosine kinase activation. We used the protein tyrosine kinase inhibitor bosutinib, which blocks Abl1 protein tyrosine kinase activation (30). As shown in Fig. 5*A*, treatment with bosutinib suppressed H_2O_2-induced MARCKS phosphorylation and PKCδ activation. siRNA-mediated knockdown of Abl1 suppressed H_2O_2-induced MARCKS phosphorylation but had no effect on basal MARCKS phosphorylation (Fig. 5*B*). H_2O_2 activation of

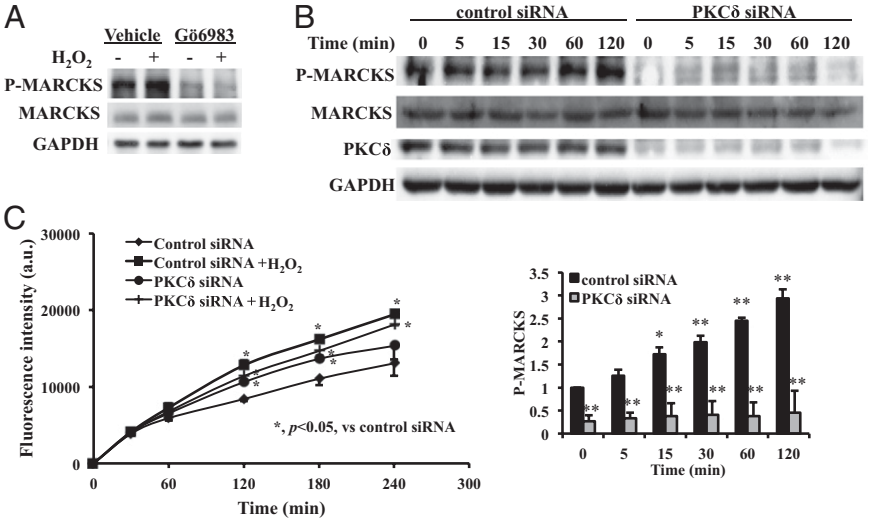

Fig. 3. PKCδ is the upstream kinase for H_2O_2-mediated MARCKS phosphorylation. (*A*) BAECs were pretreated with the PKC inhibitor Gö6983 (10 μM, 30 min) and then treated with H_2O_2 (200 μM, 30 min). Shown is a representative immunoblot of phosphorylated and total MARCKS. (*B*) BAECs were transfected with PKCδ or control siRNA, and treated with H_2O_2 (200 μM) for various times. A representative immunoblot (*Upper*) and pooled data (*Lower*) from three independent experiments are shown. **P* < 0.05; ***P* < 0.01 vs. time = 0 (solid bars) or vs. control siRNA (gray bars). (*C*) BAECs were transfected with control siRNA (diamond and square) or PKCδ siRNA (solid circle and cross), and endothelial permeability was assessed. Cells were treated with vehicle (diamond and solid circle) or with 200 μM H_2O_2 (square and cross). The plot shows the fluorescence intensity of FITC-dextran (*n* = 3). **P* < 0.05 vs. control siRNA.

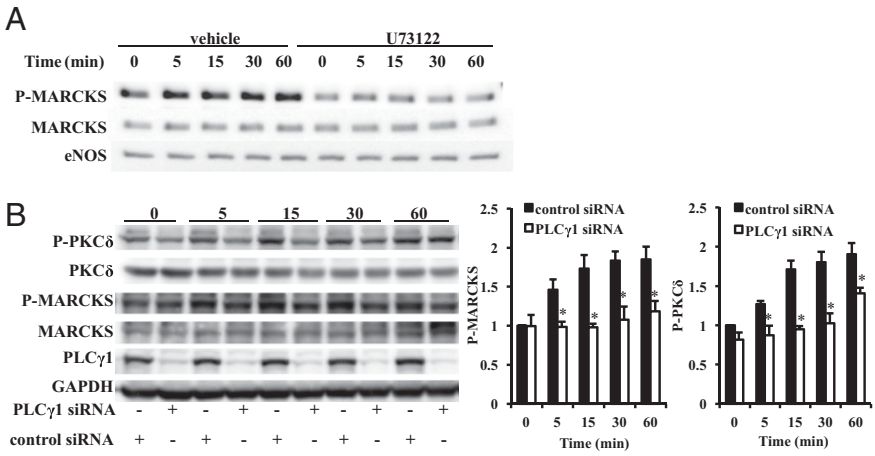

Fig. 4. PLC is involved in H_2O_2-mediated MARCKS phosphorylation. (*A*) BAECs were pretreated with the PLC inhibitor U73122 (5 μM, 30 min) and then treated with H_2O_2 (200 μM) for the indicated times. A representative immunoblot is shown. (*B*) BAECs were transfected with PLCγ1 or control siRNA, and then treated with H_2O_2 (200 μM) for the indicated times shown at the top of the figure (minutes). A representative immunoblot and the pooled data ($n = 3$) are shown. *$P < 0.05$.

both PLCγ1 and PKCδ was blocked by siRNA-mediated Abl1 knockdown (Fig. 5*B*). These results suggested that Abl11 activation is upstream of PLCγ1 and PKCδ activation in the H_2O_2-induced MARCKS phosphorylation pathway.

Rac1 Is an Early Component of the H_2O_2-Initiated Signaling Cascade That Leads to MARCKS Phosphorylation. Small GTPases are important components of H_2O_2-initiated signaling pathways (11). We have previously found that Rac1 plays a key role in endothelial signal transduction (31, 32), and we explored the role of Rac1 in H_2O_2-induced MARCKS phosphorylation. siRNA-mediated knockdown of Rac1 suppressed H_2O_2-induced MARCKS phosphorylation as well as H_2O_2-induced phosphorylation of PKCδ, PLCγ1, and Abl1 (Fig. 6). Knockdown of Rac1 had no substantive effect on basal MARCKS phosphorylation or basal PKCδ, PLCγ1, and Abl1 activation. We found that Rac1 localized mainly to the cytoplasmic region under basal conditions (Fig. S4). H_2O_2 treatment induced Rac1 translocation to the plasma membrane (Fig. S4). These results suggested that Rac1 is an early signaling component upstream of Abl1, PLCγ1, and PKCδ activation in the H_2O_2-induced MARCKS phosphorylation pathway.

Discussion

ROS are produced by a variety of vascular cells and can cause oxidative stress, influence endothelial barrier function, and modulate cytoskeletal organization (3–6). H_2O_2 is also an important physiological signaling molecule that modulates endothelial permeability (33), but the pathways involved are incompletely understood. In these studies, we have identified and characterized a role for MARCKS in a signaling cascade triggered by H_2O_2. In response to H_2O_2, BAECs transfected with control siRNA show a time- and dose-dependent increase in permeability that is mitigated by siRNA-mediated MARCKS knockdown (Fig. 1 *A* and *B*), implicating MARCKS in the H_2O_2-initiated signaling cascade leading to enhanced permeability. Prolonged exposure to H_2O_2 induced a permeability increase even in BAECs transfected with MARCKS siRNA, suggesting the involvement of additional cellular pathways controlling permeability, and indicating that MARCKS is not the sole mediator of the H_2O_2-induced permeability increase. Moreover, MARCKS knockdown does not affect basal permeability, but only the increase in permeability seen in response to H_2O_2, suggesting that the determinants of basal and H_2O_2-modulated permeability involve different cellular pathways. The regulation of microvascular permeability is an important determinant in the vasculature in vivo, but we chose to study BAECs to probe cell permeability pathways in vitro because the signaling pathways in these cells are so well characterized. It is essential to correlate these findings with studies of in vivo systems, which are stymied by the fact that the MARCKS knockout mouse is embryonic-lethal and no conditional knockouts have yet been reported. The MARCKS duplex siRNA targeting construct used

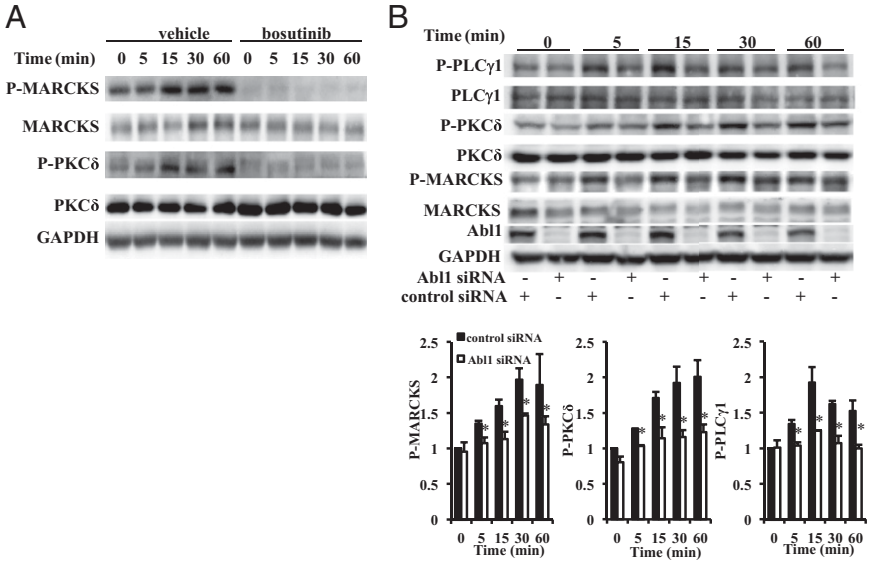

Fig. 5. Abl1 activation is necessary for H_2O_2-mediated MARCKS phosphorylation. (*A*) BAECs were pretreated with the Abl1 inhibitor bosutinib (10 μM, 30 min) and then treated with H_2O_2 (200 μM, 30 min). A representative immunoblot is shown. (*B*) BAECs were transfected with Abl1 or control siRNA, treated with H_2O_2 (200 μM) for the indicated times, and analyzed in immunoblots probed with antibodies as shown. A representative immunoblot (*Upper*) and the pooled data (*Lower*) ($n = 3$) of phospho-MARCKS, phospho-PKCδ and phospho-PLCγ1 are shown. *$P < 0.05$.

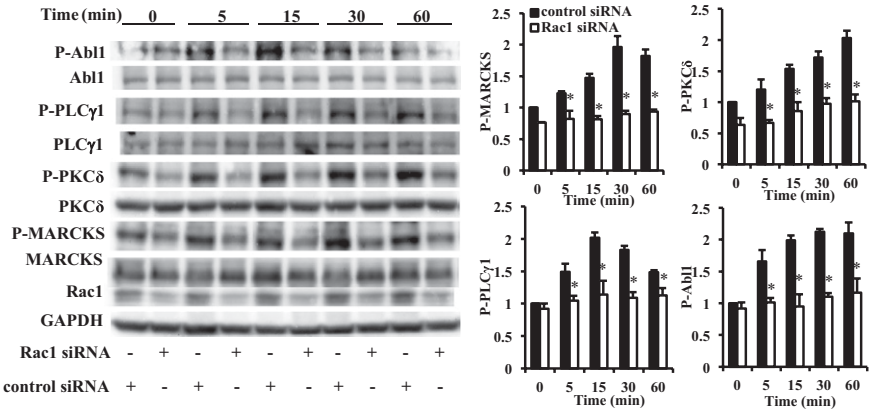

Fig. 6. Rac1 is an early component of the H_2O_2-initiated signaling cascade leading to MARCKS phosphorylation. BAECs were transfected with Rac1 or control siRNA, treated with H_2O_2 (200 μM) for the indicated times, and analyzed in immunoblots probed with antibodies against phospho-MARCKS, phospho-PKCδ, phospho-PLCγ1, phospho-Abl1, and other antibodies as shown. Panels show representative immunoblots and the pooled data from three independent experiments. $*P < 0.05$.

in these studies has been validated (20), and off-target effects seem unlikely given the specificity and potency with which this targeting construct promotes MARCKS knockdown, as revealed both in these studies and in our previous work (20). However, as with any study using RNA interference approaches, it is difficult to entirely exclude any off-target effects. In agreement with studies of human pulmonary artery endothelial cells (16), our studies in BAECs show that siRNA-mediated knockdown of MARCKS has no effect on basal cellular permeability. It is only in the case of the H_2O_2-enhanced endothelial permeability increase that MARCKS exhibits a significant effect.

Cellular permeability and cytoskeletal reorganization are intricately regulated in endothelial cells (5). Actin filaments play a key role in endothelial barrier function, yet the precise mechanism of actin modulation is unknown (34). Increases in endothelial permeability have been associated with an increase in stress fibers and a disruption of cortical actin (34). Conversely, studies of dominant-negative Rac1 in human umbilical vein endothelial cells have found increased permeability accompanied by decreased stress fiber formation, indicating that stress fiber formation is not essential for enhanced endothelial permeability (35). These studies highlight the complex nature of the role of cellular actin with respect to endothelial permeability regulation.

The proteins that regulate actin redistribution in the context of the H_2O_2-induced increase in endothelial permeability are incompletely understood. MARCKS is an actin-binding protein that modulates actin cytoskeletal organization in various cell types (14, 18). As shown in Fig. 1*C*, we saw robust actin stress fiber formation along with increased cortical actin in cells transfected with MARCKS siRNA and treated with H_2O_2. Furthermore, cells transfected with MARCKS siRNA show a sustained cortical actin structure and cell shape following treatment with cytochalasin D. Our findings indicate that H_2O_2 induces an increase in stress fiber formation, a phenomenon that is shared by MARCKS knockdown. However, MARCKS knockdown also preserves cortical actin even in the presence of H_2O_2, which may facilitate the suppression of the H_2O_2-induced permeability increase seen following siRNA-mediated MARCKS knockdown. The differences in the time courses of actin remodeling and changes in permeability may reflect the broad range of cellular responses that must be marshaled to alter cellular permeability. Clearly, the relationships between actin remodeling and endothelial permeability are complex.

Cellular migration, wound healing, and permeability may be regulated by MARCKS phosphorylation (15, 20, 36). The phosphorylation of MARCKS appears to be the fundamental molecular mechanism that determines both its subcellular localization and its interactions with key structural and signaling molecules. Agents that increase endothelial permeability, such as thrombin and diacylglycerol (37–39), also induce MARCKS phosphorylation in endothelial cells (40, 41), suggesting a link between MARCKS phosphorylation and permeability. We have characterized MARCKS phosphorylation pathways in BAECs treated with H_2O_2 and have found that H_2O_2 induces MARCKS phosphorylation and translocation from membrane to cytosol (Fig. 2). We have established a chain of signaling events—beginning with activation of Rac1 and followed by activation of Abl1, PLCγ1, and PKCδ—that result in MARCKS phosphorylation (Fig. 7). Rac1, Abl1, PLC, and PKC have all been implicated in the regulation of permeability (10, 25, 34, 35, 42, 43). Our results indicate a significant role for PKCδ-mediated MARCKS phosphorylation in the H_2O_2-induced endothelial permeability increase. In contrast to MARCKS, PKCδ also appears to modulate basal permeability, suggesting that this kinase may have an even broader role in control of vascular permeability. Taken together, these findings suggest that Rac1/Abl1/PLCγ1/PKCδ-mediated MARCKS phosphorylation may serve as an important mechanism in the regulation of the H_2O_2-induced endothelial permeability increase.

Low levels of endogenous H_2O_2 are necessary for endothelial cell proliferation and differentiation, but high H_2O_2 concentrations cause oxidative stress and endothelial dysfunction (7, 8), including alterations in endothelial permeability. Signaling molecules including PKC, PLC, Abl1, and Rho GTPase modulate endothelial permeability (10, 42–44). In addition, molecules such as PKC, Rho GTPase, and phosphatidylinositol 4,5-bisphosphate (PIP_2) regulate actin reorganization, which in turn modulates endothelial permeability. MARCKS, an actin- and PIP_2-binding protein and a substrate of PKC, is an excellent candidate mediator of actin reorganization and permeability change in endothelial cells. Here, we find that MARCKS is a unique mediator of the H_2O_2-induced endothelial permeability change and actin

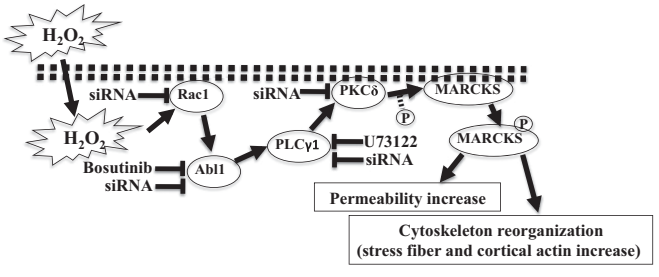

Fig. 7. A model for H_2O_2-modulated permeability increase and cytoskeleton reorganization via MARCKS phosphorylation in endothelial cells. This figure shows a model for H_2O_2 modulation of endothelial permeability and cytoskeleton rearrangement via MARCKS phosphorylation in endothelial cells. Data presented in this article indicate that H_2O_2 promotes MARCKS phosphorylation through a Rac1/Abl1/PLCγ1/PKCδ signaling pathway, associated with increased endothelial permeability and cytoskeleton reorganization. Some of the components of this pathway, such as PKCδ, may have additional roles in the modulation of basal endothelial permeability (see *Discussion*).

reorganization in endothelial cells. The MARCKS phosphorylation response involves a signaling cascade from Rac1 to Abl1, PLCγ1, and PKCδ. The MARCKS signaling cascade established in these studies may lead to the identification of candidate therapeutic targets for diseases involving altered endothelial permeability.

Materials and Methods

Materials. Reagents are described in detail in *SI Materials and Methods*. The MARCKS-GFP plasmid was a generous gift from Debbie Stumpo and Perry Blackshear (National Institute for Environmental Health Sciences).

Cell Culture, siRNA Transfection, and Immunoblotting. Cells were cultured as described previously (9, 10). Details of siRNA transfection and immunoblot analysis are described in *SI Materials and Methods*.

Immunofluorescence and Confocal Laser Scanning Fluorescence Microscopy. Single-cell imaging was performed using a Nikon TE2000 microscope with a Perkin-Elmer spinning disk confocal system at the Nikon Imaging Center at Harvard Medical School, as described in detail in *SI Materials and Methods*.

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