Endothelial Contraction and Monolayer Hyperpermeability are Regulated by Src Kinase.

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Abstract

Endothelial monolayer hyperpermeability is regulated by a myosin light chain phosphorylation (MLC-P)-dependent contractile mechanism. In this study, we tested the role of src-dependent tyrosine phosphorylation to modulate endothelial contraction and monolayer barrier function using the myosin phosphatase inhibitor, calyculin A (CalA), to directly elevate MLC-P in combination with the src-family tyrosine kinase inhibitor, herbimycin A (HA), in bovine pulmonary artery endothelial cells (EC). CalA stimulated an increase in MLC-P, src kinase activity, an increase in the tyrosine phosphorylation of paxillin and focal adhesion kinase (p125<sup>FAK</sup>), and monolayer hyperpermeability. Microscopic examination of CalA-treated EC revealed a contractile morphology characterized by peripheral contractile bands of actomyosin filaments and stress fibers linked to phosphotyrosine-containing focal adhesions (FA). These CalA-dependent events were HA-sensitive. HA alone stimulated an improvement in monolayer barrier formation by reducing the levels of MLC-P, PY-containing proteins, and the number of large paracellular holes. These data show that src kinase plays an important role to regulate monolayer hyperpermeability through adjustments in tyrosine phosphorylation, MLC-P, and EC contraction.
Introduction

The vascular endothelium provides the permeability barrier that controls the passage of fluid and solutes into the perivascular space. Inflammation of this barrier initiates a hyperpermeability state characterized by the formation of large paracellular holes between adjacent endothelial cells (EC). These events occur most frequently in the systemic post-capillary venule and pulmonary arteriole circulations. The formation of paracellular gaps are the result of reorganization of the endothelial cell-cell junctional morphology permitting the enhanced leakage of plasma proteins and fluid into the tissues causing edema and organ dysfunction. Although these changes in EC shape are controlled by cytoskeletal and cell-cell junctional proteins, the signaling mechanisms that modulate these structural events are not completely understood.

The src-family of non-receptor tyrosine kinases are known to play important roles in regulating cell growth. Recent evidence suggests that activated pp60\textsuperscript{src} (src) may adjust signaling to the cytoskeleton and cell-cell junctions (9, 16). In v-src transfected fibroblasts, where src is constitutively active and oncogenic, this kinase binds to newly formed actomyosin stress fibers, which are linked to focal adhesions (3, FA). In addition, src is recruited to filamentous (F)-actin bundles in thrombin-treated platelets (14). These data suggest that src may regulate the cytoskeletal events of EC shape change leading to vascular hyperpermeability (12, 13, 30). In this regard, inhibition of src kinase protects against the hyperpermeability consequences of stroke (23). In addition, thrombin and VEGF-induced endothelial hyperpermeability are associated with the src-dependent tyrosine phosphorylation of focal adhesion kinase (p125\textsuperscript{FAK}) and paxillin, which are concentrated at FA (6, 7, 8, 24). Since these events involve the src-dependent regulation of multiple receptor signaling cascades (17), we
studied the direct links between endothelial contraction, the actomyosin cytoskeleton, and src kinase using the myosin phosphatase inhibitor, calyculin A (CalA).

Endothelial cell contraction is due to the phosphorylation of 20-kDa myosin light chain (MLC-P) on Serine-19 and Threonine-18 (1, 13). This event initiates the formation of actomyosin bundles that attach to newly formed FA (18). The level of MLC-P is adjusted through the oppositional activities of two enzymes: a Ca\(^{2+}\)-calmodulin-dependent MLC kinase (MLCK) and myosin phosphatase (PP1M). Activation of MLCK elevates myosin ATPase activity and increases MLC-P, endothelial contraction, and monolayer hyperpermeability (1, 12, 13, 17). Although Ca\(^{2+}\)-calmodulin and cAMP-dependent regulation of MLCK is well known (1, 12), recent evidence shows that src kinase binds to MLCK and regulates its activity (4, 33). These data suggest that src kinase plays a more important role in regulating the MLCK-dependent phosphorylation of MLC than previously believed. The amount of MLC-P is also controlled by adjusting its degradation. At basal levels of intracellular Ca\(^{2+}\), the level of MLC-P is modulated by the receptor-mediated inhibition of PP1M. This so-called Ca\(^{2+}\)-sensitization of EC contraction is initiated by the activation of the small GTPase, Rho, and its kinase, ROCK (2, 6, 7, 11, 17, 19, 21, 24). Thus, src-dependent regulation of MLCK and ROCK’s inhibition of PP1M represent novel modulators of MLC-P (31, 33). In the present study, we used calyculin A (CalA), a selective inhibitor of PP1M (4, 10), as a Rho mimic to bypass the complex web of receptor-mediated second messenger signaling cascades and directly stimulate an increase in MLC-P, EC contraction, and barrier hyperpermeability.

The purpose of this study was to test the hypothesis that EC contraction and monolayer hyperpermeability were src-dependent. We used CalA to directly elevate MLC-P and actomyosin filament formation in combination with the src-specific inhibitor, herbimycin A (HA), which reverses the src phenotype in kidney cells by specific inhibition of v-src tyrosine kinase activity (15, 32). We show that the CalA-dependent tyrosine phosphorylation of p125\(^{FAK}\)
and paxillin at FA, the formation of MLC-P, EC contraction, and monolayer hyperpermeability were src-dependent. HA inhibited these CalA-induced characteristics, and HA alone stimulated a reduction in monolayer permeability. These data indicate that src-dependent reduction of EC contraction and the tyrosine phosphorylation of FA play important roles in regulating endothelial hyperpermeability.
Materials and Methods

Endothelial Cell Culture.

Our personal stock of low passage (P5-P11) endothelial cells (EC), isolated from bovine pulmonary arteries as previously described (25), were grown in DMEM containing 10% Fetal Calf Serum (Hyclone, Ogden, UT) and used between 4-5 days post-confluence.

Immunoprecipitation and Immunoblotting.

EC grown in gelatin-coated 60 mm dishes were treated with or without 1 µM herbimycin A (HA, CalBioChem, San Diego, CA) for 16 hr followed by the presence or absence of 2.5 nM calyculin A (Cala, Sigma, St. Louis, MO) for the times indicated in the text. EC were lysed and immunoprecipitated for phosphotyrosine (PY) containing proteins using 2 µg of monoclonal antibodies, PY99 (Santa Cruz Biotechnology, Santa Cruz, CA) or paxillin using anti-paxillin IgG (Transduction Laboratories, Lexington, KY) and western blotted for anti-phosphotyrosine (4G10, UpState Biotechnology, Lake Placid, NY), p125FAK, and paxillin as previously described (5). In separate experiments protein bands to activated src kinase and β-actin were detected from EC lysates by immunoblotting with phosphospecific polyclonal IgG to the Tyr416 of activated Src (Src PY416, Cell Signaling Technology) and β-actin (Sigma) using SuperSignal West Pico (Pierce, Rockford, IL). Chemiluminescence photons were detected with a Storm phosphoimager using ImageQuant software or a liquid nitrogen cooled CCD camera (Roper Scientific, Tucson, AZ) using MetaMorph Software (Universal Imaging, Bradywine, PA).

Endothelial Monolayer Barrier Function.

The techniques listed herein have been described in detail elsewhere (25, 26). In brief, the size-dependent passage of fluorescein isothiocyanate-labeled hydroxy ethyl starch (FITC-HES) macromolecules across bovine pulmonary artery endothelial cell (EC) monolayers
was used to measure the presence or absence of a small “pore” permeability barrier. FITC-HES samples from both sides of the monolayer-filter support barrier were analyzed by high-pressure size-exclusion liquid chromatography (HPSEC) and quantified with a fluorescence spectrophotometer-computer detection system (25).

EC monolayers were prepared by seeding 75 x 10³ cells onto gelatin/fibronectin coated Transwell inserts (2,000 Å pore radius filter supports, 0.33 cm² surface area; Costar, Cambridge, MA) in a 24-well plate as previously described (26). At 4-5 days post-confluence, each monolayer was incubated with 1 μM HA for 16 hr. Barrier function studies were performed by washing each Transwell with 37°C serum-free N-2-Hydroxyethylpiperazine-N′-2-Ethanesulfonic Acid (HEPES) buffered (pH = 7.4) minimum essential media (MEM). The experiment was initiated by the addition of 100 µl of FITC-HES (3 mg/ml) to the top chamber (Transwell insert) and 650 µl of serum-free HEPES-MEM to the bottom chamber (24 well plate) followed by the addition of 1 μM HA and/or 2.5 nM Cal A. Samples (30 µl) from the bottom chamber were collected at 30 min intervals and the top chamber was removed after 1.5 hr for HPLC size-separation with fluorescence quantitation. Computation of permeability/free diffusion coefficients was performed exactly as previously described (7). Monolayer experiments that did not achieve restricted diffusion in controls were excluded from the data analysis.

Digital Imaging Immunofluorescence Microscopy.

An Olympus IMT-2 microscope digital imaging workstation was used as previously described (8). In brief, a 60X 1.4 NA oil immersion objective with a Photometrics (Tucson, AZ) PXL CCD camera, a z-axis controller (Ludl Electronic Products, Ltd., Hawthorne, NY), and an Athlon XP PC with MetaMorph software 4.5 (Universal Imaging Corporation, West Chester, PA) were used to acquire epifluorescent digital images with online background-subtraction and shading correction. MetaMorph was used to identify regions of colocalization.
by color encoding the separate immunofluorescent images of each cell.

Labeling of Cytoskeletal Structures.

EC grown to 4 days post-confluence in 8 well slides were treated and stained as previously described (6) and in the text. Primary rabbit antibodies to α-catenin (1:100 dilution, Zymed Laboratories Inc.) were used to detect the adherens junctions, mouse anti-paxillin IgG (1:200 dilution, Chemicon, Temecula, CA) and anti-phosphotyrosine IgG (4G10, 1:100 dilution, UpState Biotechnology, Inc., Lake Placid, NY) were used to detect FA. After washing, secondary goat TRITC or Cy5-labeled anti-rabbit IgG (1:25 dilution) and goat-Cy5 anti-mouse IgG (1:25 dilution; Jackson ImmunoResearch Labs Inc., Westgrove, PA) were used to label each primary IgG. Alexa 488-phalloidin (Molecular Probes, Eugene, OR) was used to label F-actin. Each slide was covered with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA), a #1 coverslip was applied, and the edges were sealed with nail polish.

Myosin Light Chain Phosphorylation (MLC-P).

The phosphorylation of MLC was measured by urea/glycerol PAGE separation of the un-, mono-, and diphosphorylated forms as previously described (7, 22). In brief, EC grown in 60 mm dishes were pretreated with 1 μM of HA for 16 followed by the addition of 2.5 nM CalA for 15 min or no treatment and the reaction stopped by the addition of 1.0 ml of ice cold 10% perchloric acid. Cells were scraped, and centrifuged for 10 min at 20,000g at 4°C. The pellets were washed with 1 ml of ice cold water followed by 1 ml of ice cold ethanol and the pellet redissolved in sample buffer (6.7 M urea, 10 mM tris, 22 mM glycine, and 270 mM sucrose, pH 9.0). Equal amounts of protein were added to each lane of a 40% glycerol/10% acrylamide gel and run at 400V for ~1 hr. The protein samples were transferred to nitrocellulose in 0.25 mM phosphate buffer (pH 7.6) for ~ 1 hr. The unphosphorylated (P0), monophosphorylated (P1), and diphosphorylated (P2) forms of MLC were detected by Western blotting using a polyclonal MLC
antibody (1:1,000, James Stull, Dallas, Texas) followed by detection using ECL plus (Amersham) and Storm phosphoimager (Molecular Dynamics). The stoichiometry of MLC-P (mol phosphate/mol MLC) was determined using ImageQuant software and calculated using the formula \((P_1 + 2XP_2)/(P_0 + P_1 + P_2)\) as previously described (7).

**Statistical Analysis.**

The Student’s test (paired) was used for the statistical analysis of the results. These values are expressed as means ± SE.
Results

CalA Activates Src Kinase in an HA-Dependent Manner.

Using a site specific IgG that detects the phosphorylation of tyrosine 416 within the activation loop of src, we blotted EC lysates treated with CalA in the presence or absence of the src kinase inhibitor, HA (Figure 1). Our data show that CalA stimulated a ~25% increase in the content of PY^416 that was HA-sensitive. This effect was not due to changes in src expression or protein loading because the amount of nonphospho-src and β-actin did not change with each treatment. Since phosphorylation of PY^416 predicts an increase in enzymatic activity, these data indicate that CalA stimulates an HA-sensitive activation of src kinase.

HA Reduces EC Monolayer Solute Permeability and Inhibits CalA-Induced EC Monolayer Hyperpermeability.

We have reported that thrombin-induced formation of MLC-P and EC monolayer hyperpermeability is Rho and ROCK-dependent (6, 7). In the present study we tested whether CalA-induced formation of MLC-P and monolayer barrier dysfunction were HA-sensitive. We quantitatively measured the size and number of paracellular holes formed across CalA-stimulated EC monolayers in the presence or absence of the Src-family tyrosine kinase inhibitor, herbimycin A (HA), by analyzing their size-selective solute permeability characteristics using an HPSEC technique (24). Restricted diffusion, created by predominantly small “pores” (<250 Å pore radius), is characterized by a decline in the size-selective permeability/free diffusion coefficient (P/Do) with increasing solute molecular radius (a_e, 26). In contrast, the formation of large holes (>2,000 Å pore radius) is displayed by an increase in P/Do for solutes with large molecular radius (27). Control EC monolayers showed significant restricted diffusion, a property that was substantially enhanced by pretreatment with 1 µM HA (Fig. 2). In contrast,
CalA-stimulated monolayers displayed large hole barrier hyperpermeability as illustrated by a shift in the P/Do vs. $a_c$ curve up and to the right. HA inhibited this hyperpermeability effect.

**Cal A Stimulates EC Contraction in a HA-Sensitive Manner.**

To identify the cytoskeletal characteristics associated with how HA inhibits CalA-induced monolayer hyperpermeability, confluent EC were exposed to 2.5 nM CalA (30 min) with and without 1 µM HA pretreatment (16 hrs) followed by fixation and triplet labeling for F-actin (green), myosin II (blue), and paxillin (red). Figure 3a displays a control EC with a few F-actin stress fibers (SF, arrows) linked to paxillin-containing FA (arrowhead). CalA (Fig. 3b) stimulated a rounded, contractile morphology illustrated by multiple peripheral bands of actomyosin (blue-green) filaments (double arrowhead) suggesting an increase in centripetal tension. This behavior caused the formation of large open areas between adjacent cells (★) with a few F-actin SF (arrows) linked to paxillin-containing FA (arrowheads). HA-treated EC (Fig. 3c and d) displayed an increased formation of F-actin SF (arrows) linked to paxillin-containing FA (arrowhead). Although HA-CalA treated monolayers (Fig. 3d) displayed multiple blue-green actomyosin filaments at the cell periphery (double arrowhead), the size and number of large paracellular holes (★) were markedly reduced as compared with EC exposed to CalA alone (b).

**HA Reduces Myosin Light Chain Phosphorylation and Inhibits CalA-induced Hyperphosphorylation.**

CalA is known to stimulate the rapid phosphorylation of MLC, achieving a maximum within 15 min and initiating the EC contractile forces associated with EC monolayer hyperpermeability (10). We tested the effects of HA to prevent the CalA-induced formation of MLC-P (Fig. 4). Confluent EC were pretreated with HA (1 µM for 16 hr), followed by stimulation with 2.5 nM CalA for 15 min. The unphosphorylated ($P_0$), monophosphorylated
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(P₁), and diphosphorylated (P₂) forms of MLC were separated (Fig. 4 a), quantified by densitometry and expressed as moles of phosphate/moles of MLC (Fig. 4 b) as described in Materials and Methods. CalA stimulated the rapid incorporation of phosphate into MLC that was 2.25 fold above control. Pretreatment with 1 µM HA attenuated this CalA effect whereas 1 µM of HA alone reduced the level of MLC-P substantially (~50%) below the control levels.

**CalA Initiates a HA-Sensitive Tyrosine Phosphorylation of Paxillin and p125<sub>FAK</sub>**

Thrombin-dependent EC contraction is associated with the formation of focal adhesions at the ends of F-actin SF and the tyrosine phosphorylation of the FA proteins, paxillin, and p125<sub>FAK</sub>, characteristics inhibited by the Rho inhibitors, C3 exoenzyme and Y27632 (6, 7) and the MLC kinase inhibitor, KT5926 (29). We tested whether CalA-dependent EC contraction also stimulates the tyrosine phosphorylation of these FA proteins in a HA-sensitive manner. EC were treated with CalA (2.5 nM, 15 min) with and without HA pretreatment (1 µM for 16 hr) followed by immunoprecipitation with anti-paxillin and anti-phosphotyrosine (PY99) antibodies and western blotting with anti-phosphotyrosine (4G10), anti-p125<sub>FAK</sub>, and anti-paxillin IgG as described in the Materials and Methods. Figure 5 a-d shows that although HA did not affect the quantity of immunoprecipitated paxillin (Fig 5 a), this inhibitor caused a decline in the PY-content of two protein bands, paxillin and p125<sub>FAK</sub>, with or without CalA treatment (Fig. 5 b-d). Densitometry of the CalA-stimulated PY-containing proteins revealed an increase in the PY-content of paxillin and p125<sub>FAK</sub> (Fig. 5 d). Immunoblotting for p125<sub>FAK</sub> confirmed the identity of this band (data not shown). Since HA prevented these CalA events, the tyrosine phosphorylation of paxillin and p125<sub>FAK</sub> appear to be src-dependent.

**PY-Containing Proteins and Large Hole Formation.**

Finally, we tested whether HA inhibited the CalA-induced formation of PY-containing FA and large paracellular holes. EC monolayers pretreated with and without HA (1 µM for 16
hr) followed by stimulation with and without CalA (2.5 nM for 0.5 hr) were triple stained for F-actin, \( \alpha \)-catenin, and PY, and digital images were collected. Figure 6 a-d shows a control EC displaying F-actin SF (arrows, \( a \), insert) linked to a few PY-stained FA (arrowheads, \( c \), insert) with a rim of \( \alpha \)-catenin (\( b \)). The tricolor overlay displays that PY-containing FA (arrowheads) are linked to the ends of F-actin stress fibers (SF, arrows, insert). Figure 6 e-h illustrates the effects of HA to increase F-actin SF (arrows, \( e \)) but reduce the content of PY-containing FA (arrowheads, \( g \)). This HA-dependent loss of yellow-red PY staining at FA is revealed by comparing the tricolor overlay inserts of the control (\( d \)) and the HA-treated (\( h \)) EC. Figure 7 a-d shows that CalA caused the formation of large paracellular holes (\( \bullet \), in \( a, b, d \)) in association with increased F-actin SF (arrows, \( a, d \)) linked to enlarged PY-containing FA (arrowheads, \( c \)). The colocalization of PY-containing (red) FA at the ends of F-actin stress fibers are shown in \( d \) (insert). Figure 7 e-h illustrates that HA prevented this CalA effect, as revealed by the loss of PY staining (arrowheads, \( g, h \), insert) at the ends of SF (arrows, \( e, h \)) as compared with the CalA control (arrowheads, \( d \), insert).
Discussion

In the present study, we provide evidence for the first time that src kinase-dependent tyrosine phosphorylation plays an important role in regulating EC monolayer barrier function. Using CalA to directly elevate MLC-P and EC contraction, we found that these events activated src kinase-dependent tyrosine phosphorylation of the FA-containing proteins, p125^{FAK} and paxillin. These biochemical events stimulated monolayer hyperpermeability characterized by rounded EC, which showed multiple peripheral actomyosin contractile bands and enlarged spaces between adjacent cells. Since these CalA-dependent characteristics were inhibited by HA, they appear to be src-dependent. In addition, HA alone selectively reduced monolayer permeability to small solutes (\(a_e < 30 \text{ Å}\)) by reducing the number of large paracellular holes as previously observed after treatment with cAMP or the MLCK inhibitor, KT5926 (28, 29). This HA-induced improvement in endothelial barrier function was associated with the loss of src kinase activity and reduced amounts of MLC-P and PY-containing substrates. Immunostaining of HA-treated EC revealed an increase in the number of stress fibers linked to paxillin-containing focal adhesions (FA), but with reduced levels of PY-containing proteins. Taken together, our data suggest that src kinase plays an important role in the modulation of EC barrier function by adjusting MLC-P and the PY-content of FA. Since HA does not completely prevent CalA-induced paracellular hole formation and monolayer hyperpermeability, this process may be partially mediated by src-independent, non-cytoskeletal forces.

A role for the src family of non-receptor tyrosine kinases in the control of cellular function is not new. The infection of fibroblasts with v-src, the oncogenic product of Rous sarcoma virus that is constitutively active, leads to increased cell migration and growth in association with src binding to the cytoskeleton (3, 18). Recent evidence shows that src not only regulates the activation of receptor tyrosine kinases, but also plays an important role in G-
protein-coupled receptor-mediated signal transduction (16). Previous work has identified that src-dependent tyrosine phosphorylation is a critical requirement for the functional formation of integrin-dependent FA attachment to F-actin stress fibers (20). In the present study we extend these data to show that src also regulates endothelial monolayer permeability at the cytoskeletal level by adjusting the content of both MLC-P and the PY-containing FA proteins, paxillin and p125<sup>FAK</sup>. To confirm src’s role in CalA-induced EC cytoskeletal signaling, we show that the selective src kinase inhibitor, HA (15, 32), prevents the CalA-dependent activation of src kinase and limits the formation of MLC-P. HA alone reduced the level of MLC-P to nearly 50% of the control EC. These data are consistent with a recent report where src regulated MLCK activity, in vitro (4). In addition, our data is consistent with the notion that by reducing the PY content at FA, HA inhibits the hyperpermeability effects of CalA. Taken together, these data support a role for src to regulate EC contraction and monolayer solute permeability through the inhibition of MLC-P and the tyrosine phosphorylation of the FA proteins, paxillin and p125<sup>FAK</sup>.

How CalA stimulates the activation of src kinase is not clear. CalA is a Rho mimic (4, 10) that elevates the levels of MLC-P by inhibiting the catalytic activity of PP1M (33). This phosphatase inhibitor has been previously used to increase the formation of MLC-P and actomyosin filaments and elevate monolayer permeability (10). Since increased amounts of MLC-P stimulate actomyosin filament formation, this CalA effect may recruit src to the F-actin cytoskeleton as previously shown in v-src transformed cells (3). Src contains two important regulatory tyrosine phosphorylation sites. Phosphorylation at tyrosine 527 in the carboxyl tail decreases kinase activity, while phosphorylation within the activation domain at tyrosine 416 (Tyr-416) positively regulates src kinase activity (16). Using an antibody that detects the amount of phosphorylated Tyr-416 in src, we show that the CalA-dependent elevation in MLC-P, and actomyosin filaments linked to newly formed FA were associated with increased src kinase
activity. Taken together, these data suggest a link between the activation of src kinase and the formation of the actomyosin cytoskeleton.

Thrombin stimulates actomyosin-based SF contractile forces, causing cell retraction and rounding in EC and neurites (12, 13, 17). We have shown that this process is mediated by Rho and ROCK (6, 7). In the present study, we confirm separate report that CalA elevates monolayer hyperpermeability by stimulating EC contraction (10). However, these authors showed that although the MLC kinase inhibitor, KT5926, prevented MLC-P formation, it did not block monolayer hyperpermeability. These data suggested that changes in cell-cell adhesion may be involved in the process. In the present study, although HA limited the CalA-dependent increase in the phosphorylation of MLC, these treatments did not affect the formation of F-actin SF. In addition, HA did not completely prevent CalA-induced large hole formation. These data suggest that CalA-induced monolayer hyperpermeability is only partially dependent on MLC-P and actomyosin SF formation. Taken together, CalA-induced monolayer barrier dysfunction appears to involve both the phosphorylation of MLC leading to EC contraction and other non-cytoskeletal mechanisms.

In conclusion, our results confirm recent reports that the inhibition of PP1M acts as an important mediator to regulate cytoskeletal contraction and EC monolayer hyperpermeability. CalA-dependent increases in EC contraction and monolayer hyperpermeability were associated with increased src kinase activity and tyrosine phosphorylation of PY-containing FA. Our finding that HA alone stimulates reduced monolayer solute permeability in association with a reduction in large paracellular holes, indicates an important role for src-dependent tyrosine phosphorylation in the modulation of EC barrier function. This process appears due to src-dependent adjustment of MLC-P and the PY content of FA at the ends of SF.
Acknowledgments

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Figure Legends

Figure 1. CalA Activates Src in an HA-Sensitive Manner. Western blots of activated Src kinase as compared with β-actin are shown. EC monolayers treated with or without HA (1 µM for 16 hr) were stimulated with CalA (2.5 nM) followed by cell lysis. Src kinase activity was estimated by immunoblotting for active Src using anti-Tyr416 Src IgG in comparison with β-actin as the loading control as described in the Materials and Methods. CalA stimulated a time-dependent increase in Src kinase activity that was blocked by HA pretreatment. The relative change in Src kinase activity is also displayed for each treatment.

Figure 2. HA Reduces Monolayer Solute Permeability and Inhibits CalA-Induced Hyperpermeability. Permeability/free diffusion coefficients (D_o) vs. solute molecular radius (a_e) curves are shown. Control monolayers exhibited restricted diffusion, characterized by a polynomial decline in this curve. Herbimycin A (1 µM, HA for 16 h) caused a substantial reduction in monolayer solute permeability for all a_e. In contrast, Cal A (2.5 nM for 60 min) caused an increase in monolayer permeability characterized by the formation of large paracellular holes as indicated by shift in this curve up and to the right. This characteristic was inhibited in the HA (1 µM for 16 h)-CalA (2.5 nM for 60 min) group. Values depicted represent the mean ± SE of six separate determinations.

Figure 3. HA Initiates the Formation of F-actin SF/FA and Inhibits CalA-Induced Large Hole Formation. Wide field tricolor overlay images of EC monolayers stained for (F)-actin (green), non-muscle myosin II (blue), and paxillin (red) after exposure to CalA (2.5 µM for 30 min) with and without HA (1 µM for 16 hrs) are shown. a) Control EC displayed a few F-actin
SF (arrow) linked to paxillin-containing FA (arrowhead). b) CalA-treated cells showed a contracted morphology with increased F-actin SF (arrow) and large open areas (※) between adjacent EC. c) HA-treated EC revealed an increase in the number of F-actin SF (arrow) linked to paxillin-containing FA (arrowhead). d) HA-CalA-treated EC display increase in SF (arrow) with paxillin-containing FA (arrowhead) but reduced paracellular hole formation (※) in comparison with the Cal A-treated EC (b). Bars = 3 µm

Figure 4. HA Inhibits but Does Not Prevent CalA-Induced Myosin Light Chain Phosphorylation.

EC monolayers with or without herbimycin A pretreatment (HA, 1 µM for 16 hr) were stimulated with calyculin A (CalA, 2.5 nM for 15 min) followed by measurement of the phosphorylation of myosin light chain (MLC) as described in the Materials and Methods. (A) A representative Western blot of the unphosphorylated (P₀), monophosphorylated (P₁), and diphosphorylated (P₂) forms of MLC observed in control, HA, CalA, HA+CalA treated EC monolayers is shown. (B) The results shown in (a) are displayed as mol phosphate/mol of MLC.

Figure 5. HA Reduces Paxillin and p125FAK Tyrosine Phosphorylation. EC monolayers were pretreated with HA (1 µM for 16 h) with or without calyculin A (CalA, 2.5 nM for 15 min) or were not treated (IgG control). EC lysates were immunoprecipitated (IP) with anti-paxillin or anti-PY (PY99) IgG, SDS-PAGE chromatographed and western blotted (IB) for paxillin, p125FAK, or PY-containing proteins with use of 4G10 antibodies (a-c). Densitometry revealed that relative PY content of paxillin and p125FAK was increased by CalA but reduced in HA-treated EC as compared with control (d).
Figure 6. HA Stimulates F-actin Stress Fibers with Reduced Tyrosine Phosphorylation at Focal Adhesions. EC monolayers pretreated with herbimycin A (HA, 1 µM for 16 hr) as compared with no treatment (control) were fixed, extracted, and stained for F-actin, α-catenin, and tyrosine phosphorylated (PY) proteins as described in the Materials and Methods and confocal images displayed. Control EC displayed F-actin SF (arrows, a, d) linked to PY-containing proteins at FA (arrowheads, c, d, insert) with α-catenin at cell-cell adhesions (b). HA stimulated the formation of F-actin SF (arrows, e, h, inserts) and the loss of PY-containing proteins at FA (arrowheads, g, h, inserts) and reorganized α-catenin (f, insert). Bar = 5 µm.

Figure 7. HA Reduced Tyrosine Phosphorylation and Prevents CalA-Induced Large Hole Formation. EC monolayers were treated with CalA (2.5 µM for 30 min) in the presence or absence of HA pretreatment (1 µM for 16 hr) followed by fixation and staining for F-actin, α-catenin, and tyrosine phosphorylated (PY) proteins as described in the Materials and Methods. CalA-treated EC displayed the formation of large paracellular holes (*, a, b, d, inserts) and F-actin SF (arrows, a, g), linked to PY-containing proteins at the FA (arrowheads, c, d, insert). HA-CalA treated EC showed increased F-actin SF (arrows, e, h), with the absence of PY-containing proteins at FA (arrowheads, g, h, insert). Image scale is the same as in Fig. 5.
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