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Vascular Biology, Atherosclerosis and Endothelium Biology

Pericyte Rho GTPase Mediates Both Pericyte Contractile Phenotype and Capillary Endothelial Growth State

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Pericytes regulate microvascular development and maturation through the control of endothelial cell motility, proliferation, and differentiation. The Rho GTPases have recently been described as key regulators of pericyte shape and contractile phenotype by signaling through the actin cytoskeleton in an isoactin-specific manner. In this report, we reveal that Rho GTPase-dependent signal transduction not only influences pericyte shape and contractile potential but also modulates capillary endothelial proliferative status and pericyte-endothelial interactions *in vitro*. We provide evidence that overexpression of mutant Rho GTPases, but not other Ras-related small GTPases, significantly alters pericyte shape, contractility, and endothelial growth state in microvascular cell co-cultures. In particular, we describe the use of a silicon substrate deformation assay to demonstrate that pericyte contractility is Rho GTP- and Rho kinase-dependent; further, we describe a novel *in vitro* system for examining pericyte-mediated endothelial growth arrest and show that control pericytes are capable of growth-arresting capillary endothelial cells in a cell contact-dependent manner, whereas pericytes overexpressing dominant-active and -negative Rho GTPase are comparably incompetent. These data strongly suggest that signaling through the pericyte Rho GTPase pathway may provide critical cues to the processes of microvascular stabilization, maturation, and contractility during development and disease. *(Am J Pathol 2007, 171:693–701; DOI: 10.2353/ajpath.2007.070102)*

Development, maturation, and remodeling of the vascular system is a multistage process with regulatory mechanisms at each step.[1] Several perivascular cell types play major roles in the modulation of microvascular maturation and contractility, including the smooth muscle cells associated with arteries and the pericytes associated with venules and capillaries.[2,3] Perivascular cell regulation of the capillary microenvironment occurs through dynamic maintenance of the basement membrane as well as regulation of microvascular tone, through a complex array of signaling intermediates.[4] A complete understanding of vascular development, the physiology of capillary tone, and the regulation of capillary permeability provides insight into the pathophysiology of the vascular dysfunction associated with tumor angiogenesis,[5] age-related macular degeneration,[6] and diabetic retinopathy,[7] as well as the physiological angiogenesis of wound healing.[8]

The microvascular pericyte in particular has been the subject of considerable experimental interest because of its role in regulation of microvascular endothelial growth and differentiation[9] as well as capillary contractility and microvascular tone.[10] In particular, through both pericyte-endothelial cell contact-dependent as well as endothelial-independent mechanisms, pericytes have been postulated to govern the phenotypic change from a proliferative angiogenic sprout to a mature microvascular conduit with a quiescent capillary endothelium.[11,12] Both direct evidence for pericyte suppression of endothelial growth[13] and migration[14] as well as *in situ* correlation between pericyte investment and vessel stability have been reported.[11,15] Interestingly, pericyte investment has been implicated in conferring capillary stability and resistance to regression *in vivo*,[16,17] suggesting that pathological angiogenesis requires previously quiescent endothelium to destabilize its association with pericytes and reenter the cell cycle. Although recent work in tumor biology has also highlighted pericytes as a novel drug

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target that may enhance the efficacy of anti-angiogenic chemotherapy,[18–21] the precise molecular and biochemical mediators regulating pericyte-dependent microvascular remodeling remain equivocal.

Evidence is accumulating that vascular morphogenesis may be regulated by members of the Rho family of small GTPases.[22] Upstream of multiple cytoskeletal and kinase-mediated effectors, Rho GTPases control physiological maintenance of arterial tone, as well as the dysregulation and hypertrophic remodeling associated with essential hypertension.[23,24] Importantly, a recent role for Rho GTPase-dependent signal transduction has now been suggested in the control of pericyte shape and contractility, leading to microvascular tone and blood flow regulation via alterations in cytoskeletal dynamics.[25]

In this study, we demonstrate that pericyte-specific and Rho GTPase-dependent signal transduction reversibly regulates both pericyte contractility and capillary endothelial cell growth state. Building on the previous work of Harris et al[26] and others, we have developed two novel *in vitro* systems to directly quantify and simultaneously link the contractile potential of microvascular pericytes with pericyte Rho GTPase-mediated endothelial cell growth control. In these systems, we alter pericyte Rho GTPase expression via both adenoviral-mediated gene delivery and direct transfection of dominant-active or -negative Rho constructs. Results reveal that increased signaling through the Rho GTPase pathway significantly augments pericyte contractility and impairs pericyte efficacy in inducing endothelial cell growth arrest through both contact-dependent and contact-independent pericyte-endothelial interactions. Therefore, alterations in Rho GTPase-dependent signal transduction specifically modulate pericyte shape and contractile phenotype, as well as regulate their ability to control endothelial growth. This lends support for the notion that pathological angiogenesis is linked to alterations in endothelial growth state downstream of signaling aberrations within microvascular pericytes.

Materials and Methods

Cell Culture

Bovine retinal pericytes (expressing vascular smooth muscle actin, NG2 proteoglycan, and 3G5) and endothelial cells (expressing CD31, von Willebrand factor, and demonstrating uptake of acetylated low-density lipoprotein) were isolated from neonatal cow retina as previously described[27] and used through passage three on tissue culture-treated plasticware (Corning, Inc., Corning, NY) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% bovine calf serum (Hyclone, Logan, UT), supplemented with penicillin, streptomycin, and Fungizone (Invitrogen). Cells were grown in 24-well tissue culture plates (Corning, Inc.) in a total volume of 1 ml unless otherwise noted.

Recombinant Adenoviruses and Infection

Adenoviruses expressing dominant-active and dominant-negative Rho GTPase under the control of a tetracycline transactivator were obtained from Daniel Kalman (Emory University School of Medicine, Atlanta, GA). The viruses were amplified in human embryonic kidney 293 cells and purified by freeze/thaw and centrifugation. Expression of each virus was tested by infection of COS7 cells for 12 hours at multiplicities of infection of 100 to 500 followed by immunoblot of cell lysates and immunofluorescence microscopy with anti-Rho antibodies (clone 26C4; Santa Cruz Biotechnology, Santa Cruz, CA; data not shown). In the experiments detailed here, pericytes were infected with dominant-active or dominant-negative Rho GTPase-containing viruses in combination with the transactivator virus in serum-containing media for 6 hours at optical density-determined multiplicities of infection of 216, 298, and 286 for dominant-active Rho, dominant-negative Rho, and tetracycline transactivator-containing virus, respectively.

Plasmids and Transfection

Dominant-active Ras in vector pZipNeo (pZipNeo-RasL61) was the generous gift of Dr. Deniz Toksoz (Tufts University School of Medicine, Boston, MA). Dominant-active Rac1 (pMT3RacL61) and dominant-active Cdc42 (pMT3Cdc42L61) in vector pMT3 were contributed by Dr. Larry Feig (Tufts University School of Medicine, Boston, MA). Green fluorescent protein (GFP)-expressing plasmid (pEGFP-N3) was purchased from Clontech (Palo Alto, CA). Pericytes were transfected with 0.8 μg of DNA per coverslip for 24 hours per the Effectene transfection reagent protocol ($n > 6$ for each condition; Qiagen, Valencia, CA).

Rho GTPase Small Molecule Inhibitor

The pyridine derivative (*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632; Sigma-Aldrich, St. Louis, MO), previously shown to specifically inhibit the activity of p160 Rho-associated kinase at an IC_{50} of 140 nmol/L,[28] was resuspended in sterile water, stored at 10 mmol/L, and used at final concentrations ranging from 1 to 10 μmol/L.

Pericyte-Endothelial Cell Co-Culture Assay

Co-Culture

Pericytes were plated onto UV-sterilized 12-mm circular glass coverslips (Fisher, Pittsburgh, PA) and placed in 24-well tissue culture plates (Corning, Inc.) at a density of 10,000 cells/well in complete 10% bovine calf serum containing DMEM for 24 hours, followed by infection with recombinant adenovirus at multiplicities of infection of 200 to 300 as above in complete 10% bovine calf serum containing DMEM for 6 hours. After infection, cells were washed and incubated for an additional 18 hours before the addition

of 10,000 cells/well of freshly trypsinized bovine retinal endothelial cells in complete 10% bovine calf serum containing DMEM. After 20 hours of co-culture, media was supplemented with 10 μg/ml 5′-bromodeoxyuridine (BrdU; Sigma-Aldrich). At 24 hours of total co-culture, cells were prepared for immunocytochemistry.

Immunocytochemistry

Coverslip-attached co-cultures were fixed in 4% paraformaldehyde containing serum-free DMEM for 5 minutes at room temperature. Cells were permeabilized for 90 seconds at room temperature in freshly prepared 50 mmol/L HEPES/50 mmol/L PIPES buffer supplemented with 1 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 75 mmol/L KCl, and 0.1% Triton X-100. Chromosomally incorporated BrdU epitopes were unmasked by incubation for 15 minutes at 37°C in 1 N HCl in phosphate-buffered saline (PBS). Primary antibody incubation was performed at 4°C in a humidified chamber overnight; fluorophore-tagged secondary antibody incubation was performed at room temperature in a humidified chamber for 45 minutes. Nuclei were counterstained with 1:1000 Hoechst no. 33258 (Sigma-Aldrich) in PBS.

Antibodies

Staining was performed using anti-smooth muscle actin (SMA) (clone 1A4; Biogenex Laboratories, San Ramon, CA), anti-BrdU (BD Pharmingen, San Diego, CA), and anti-Myc (clone A14; Santa Cruz Biotechnology) primary antibodies and Alexa Fluor 488- and Alexa Fluor 546-conjugated secondary antibodies (Invitrogen) diluted 1:200 in PBS/azide. Visualization was performed using an Axiovert 200M microscope (Carl Zeiss, Thornwood, NY) at ×400, and image analysis was performed with Metamorph software (Universal Imaging Corp., Downingtown, PA).

Quantification

Pericytes were identified by immunoreactivity for SMA; dominant-active Rho GTPase-infected pericytes were identified by additional immunoreactivity for a Myc epitope tag, whereas dominant-negative Rho GTPase-infected pericytes were identified based on previously described morphology.[25] In particular, Rho-inhibited pericytes possess a highly spread-out morphology with enlarged surface area, as well a characteristic radial pattern of phalloidin-staining with markedly diffuse central vascular smooth muscle actin staining. Contact between pericytes and endothelial cells was confirmed at ×630 magnification with high-resolution optics (NA = 1.4). Co-cultures were assessed for endothelial proliferative index by examining pericyte-contacting versus lone SMA-negative endothelial cells and scoring each subset for proliferating versus nonproliferating cells assessed by nuclear BrdU immunoreactivity. Observers scored experiments by alphanumeric code, blinded to experimental condition. The number of proliferating individual endothelial cells in contact with processes from a single pericyte was scored and compared with lone endothelial cells with no cell-cell contacts; endothelial cells in contact with other endothelial cells or multiple pericytes as well as endothelial cells with pyknotic or atypical nuclei were excluded from analysis. Endothelial proliferative index was calculated as the percentage of BrdU-positive endothelial cells versus total endothelial cells in each subset. Experiments were performed at least three times in triplicate, with >300 cells assessed per condition; results are expressed as mean percentages ± SE.

Analysis of Pericyte Contractile Phenotype

Preparation of Deformable Silicone Substrates

Deformable silicone substrates were prepared essentially as previously described.[26] In brief, 20 to 50 μl of dimethylpolysiloxane (no. 123K0769; Sigma-Aldrich) were pipetted onto 35-mm round glass coverslips using a positive displacement pipettor. The silicone substrate was permitted to spread at room temperature before heat cross-linking by passing the silicone-coated coverslip through a Bunsen burner flame. Silicone-coated coverslips were then placed within a glow discharge apparatus,[29] generating a plasma discharge onto the silicone to create a hydrophilic surface permitting protein adsorption. Coated coverslips were then incubated with 0.1% collagen type I in PBS to facilitate subsequent cell attachment.

Quantitation of Contractile Force Production in Pericytes

Pericytes expressing virally transduced mutant Rho GTPases or empty vector control plasmids as well as cells treated with pharmacological inhibitors were plated on deformable silicone substrates after viral infection or as a function of drug treatment as described above. Silicone-attached pericytes were grown in cell culture chambers enabling live cell viewing for protracted time periods. The rate and extent of silicone substrate deformation was then quantified using standard morphometric analyses applied to phase-contrast images taken using a Zeiss Axiovert 200M computer-assisted light microscope imaging workstation. Each experiment was performed at least three times in triplicate; mean percentages of cells wrinkling ± SE were graphed as a function of Rho GTPase and Rho kinase inhibitor treatments.

Results

Alteration of Rho GTPase Signaling in Pericyte Cell Culture

In vivo, pericytes regulate microvascular dynamics through multiple different mechanical and biochemical pathways; however, these pathways are as yet poorly elucidated. To more closely examine the role that the Rho GTPase signaling cascade plays in pericyte function and

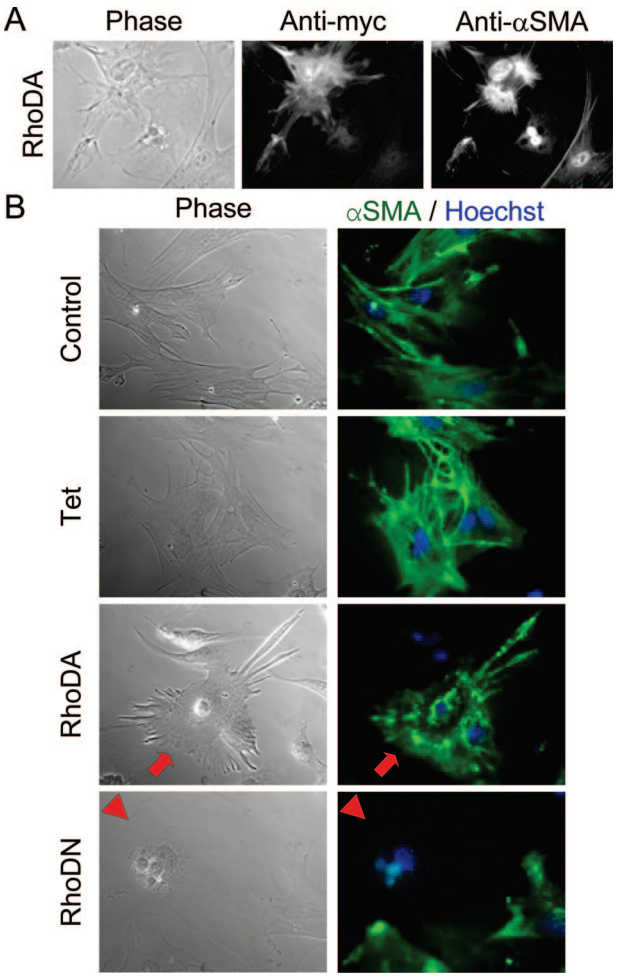

Figure 1. Adenoviral alteration of Rho GTPase signaling modulates pericyte morphology. **A:** Bovine retinal pericytes were incubated for 24 hours, co-infected with adenoviruses containing dominant-active Rho GTPase under the control of a tetracycline-repressible transactivator for 6 hours, washed with fresh media, and incubated for an additional 18 hours. Cultures were then fixed and stained for the presence of the Myc epitope tag and for α-smooth muscle actin with parallel phase images as labeled. **B:** Bovine retinal pericytes were infected as above with adenoviruses containing control tetracycline-repressible transactivator (Tet) alone, dominant-active Rho GTPase and Tet (RhoDA), or dominant-negative Rho GTPase and Tet (RhoDN), or mock-infected (control). Cultures were then fixed and stained for the presence of α-smooth muscle actin (green), with Hoechst nuclear counterstaining (blue), with parallel phase images. **Arrows** indicate a stably infected RhoDA pericyte; **arrowheads** indicate a stably infected RhoDN pericyte. Original magnifications: ×200 (**A**); ×100 (**B**).

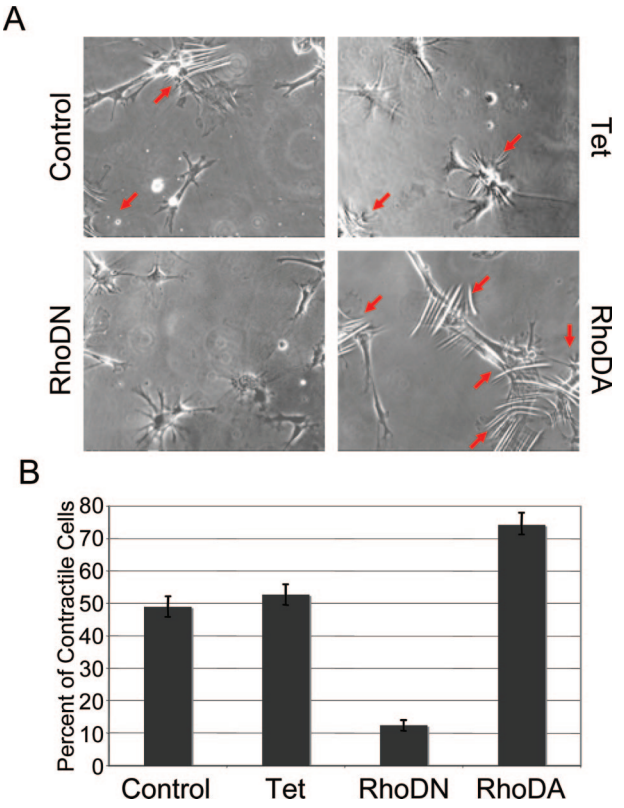

Figure 2. Adenoviral alteration of Rho GTPase signaling modulates pericyte contractility. **A:** Bovine retinal pericytes were transduced with adenoviruses containing dominant-active (RhoDA) or dominant-negative (RhoDN) Rho GTPase mutants, tetracycline-repressible transactivator (Tet) alone, or mock-infected (control). After incubation for 24 hours after infection, cells were trypsinized and replated onto plasma glow discharge-prepared, type I collagen-coated silicon substrates. Cultures were monitored in a modified cell culture container allowing real-time phase-contrast imaging during culture. Representative phase-contrast images are provided as labeled, where **arrows** indicate substrate-wrinkling, actively contractile pericytes. Original magnifications, ×400. **B:** At 24 hours, contractility was assessed by the number of pericytes producing visible substrate wrinkling per each condition, expressed as mean percentages ± SE ($n > 100$ cells per condition, triplicate experiments.

microvascular physiology, we used both adenovirus-based overexpression of dominant-active or dominant-negative Rho GTPase mutants in conjunction with a specific small molecule inhibitor to alter signaling through Rho GTPase and its downstream effector, Rho kinase. Not only were the downstream effects of mutant Rho GTPase overexpression characterized, but Rho GTPase-overexpressing and Rho kinase-inhibited pericytes were assayed for pericyte contractile phenotype and endothelial proliferation. Results of these experiments reveal that perturbation of the Rho GTPase signaling pathways significantly influences pericyte contractile phenotype, as well as endothelial cell growth state.

Previous work indicated that transient transfection of mutant Rho GTPases alters pericyte cytoskeletal array and cell shape by signaling through the actin network in an isoform-specific manner.[25] To expand on this, we used a drug-selectable, adenoviral-mediated gene delivery system to specifically induce mutant Rho GTPase expression in microvascular pericytes. Dominant-active (Myc-tagged) and dominant-negative (untagged) Rho GTPase expression was controlled using an adenoviral delivery system under the control of a tetracycline-repressible promoter (Tet).[30,31] Pericytes were plated and allowed to adhere for 24 hours before infection with altered Rho GTPase constructs; after a second 24-hour period of growth, cells were fixed and stained for α-SMA. As shown in Figure 1, pericytes expressing the constitutively active (GTP-bound) form of Rho GTPase (RhoDA) as verified by immunoreactivity for the Myc epitope tag seem hypercontractile, containing numerous actin-enriched projections surrounding a centrally contracted cytoplasmic mass [Figure 1, A (arrows) and B]. This is consistent with previously published observations using transient transfection of altered Rho GTPase constructs, reporting smaller average cell size as well as formation of

pronounced stress fibers.[25] In contrast, pericytes transduced with a dominant-negative Rho GTPase (irreversibly locked in an inactive GDP-bound form, RhoDN) appear as flattened and polygonally shaped cells (Figure 1B), with a γ-actin-containing cytoskeleton unaffected by the GDP-bound Rho GTPase overexpression; this is consistent with previous published characterization of the dominant-negative Rho phenotype as possessing significantly larger average cell size and a cytoskeletal architecture rich in phalloidin-staining stress fibers.[25] This morphology is easily distinguished from the reduced surface area, condensed shape, and diffuse attenuated expression of α-SMA observed in the dominant-active Rho GTPase-transduced cell cultures (Figure 1, A and B). Tetracycline transactivator-infected (Tet) and control uninfected pericytes retain the characteristic wild-type morphology, with isoactin cytoskeletons harboring significant α-SMA stress fiber expression (Figure 1B).

Rho GTPase Signaling Control of Pericyte Contractile Phenotype

Given the morphological differences between dominant-active and -negative Rho GTPase-expressing pericytes, we hypothesized that these differences represent a Rho-dependent change in contractile potential as well. We quantified contractile force production by using a cell culture-treated deformable silicon substrate as described in detail in the Materials and Methods section. Dominant-active (RhoDA), dominant-negative (RhoDN), or control-infected pericytes (Tet) as well as noninfected wild-type pericytes (control) plated on these silicone substrata were mounted into a temperature-controlled cell culture chamber permitting light microscopic visualization during culture. Force transduction was monitored dynamically by quantifying the percentage of cells able to generate sufficient force to produce substrate deformation visible by light microscopy (Figure 2, arrowheads indicate wrinkle-producing contractile cells). Consistent with the above-described morphological data, dominant-active Rho-infected pericytes demonstrated a contractile, substrate-deforming phenotype with 1.5-fold greater frequency compared with vector-infected and uninfected pericytes (RhoDA 74.12 $\pm$ 3.13%, $P < 0.05$ compared with either Tet or control). Conversely, dominant-negative Rho-infected pericytes generated sufficient contractile force to produce a substrate-deforming phenotype at 25% of the control frequency (RhoDN 12.4 $\pm$ 1.81%, $P < 0.05$ compared with either Tet or control). Vector alone-infected pericytes were similar to uninfected controls, with baseline contractile frequencies of 52.66 $\pm$ 3.51% and 48.98 $\pm$ 3.48%, respectively.

To localize more finely the downstream effectors responsible for contractile phenotype regulation in Rho GTPase-altered pericytes, we treated stably infected cells with the small molecule Rho kinase inhibitor Y-27632[28,32] and reassessed their contractile state (Figure 3). Consistent with Rho GTPase control of contractile phenotype in wild-type pericytes, substrate-wrinkling frequency was reduced from baseline to complete extinction throughout the micromolar range of Y-27632 concentration in a dose-dependent manner (Figure 3, arrows indicate wild-type wrinkle-producing contractile cells). Whereas 8 μmol/L Y-27632 is required to completely abolish contractile activity in pericytes overexpressing dominant-active Rho, 50% less Y-27632 (4 μmol/L) is sufficient to extinguish contractility in dominant-negative Rho-expressing pericytes. Indeed, treatment of dominant-active Rho-expressing pericytes with Y-27632 completely abrogates the hypercontractile state in a dose-dependent manner. The Y-27632 dose required to inhibit the steady state contractility of wild-type and vector-transduced pericytes is equivalent (Figure 3).

Rho GTPase Signaling Control of Pericyte-Mediated Endothelial Cell Growth Arrest

In addition to revealing the role that Rho GTPase signaling plays in controlling pericyte shape and contractile phenotype, we further investigated whether perturbations in Rho GTPase-dependent signal transduction are similarly instrumental in endothelial growth control. To determine whether perturbation of the Rho GTPase signaling pathway altered the pericyte's ability to modulate capillary endothelial growth state, we developed a co-culture assay in which contact-dependent and -independent pericyte-mediated endothelial growth control could be quantitatively assessed. In this assay, Rho-altered pericytes are co-cultured with capillary endothelial cells; cell-cell contact and proliferative status are quantified, allowing assessment of the effects of both pericyte contact and pericyte-derived soluble mediators on endothelial proliferation (Figure 4A).

In parallel with control of pericyte morphology and contractility, alterations in Rho GTPase signaling also caused significant abrogation of pericyte-mediated endothelial growth arrest (Figure 4B). At baseline, control and uninfected pericytes demonstrated significant inhibition of proliferation in pericyte-contacting versus lone endothelial cells by 49.9 $\pm$ 13.5% and 44.0 $\pm$ 9.7%, respectively ($n = 636$ and 719, $P < 0.05$ for both conditions). In comparison, both dominant-active and -negative Rho-expressing pericytes failed significantly to contact-inhibit adjacent endothelial cells, with contacted endothelial cell proliferation rates 1.22 $\pm$ 0.08 and 1.28 $\pm$ 0.09 times that of lone endothelial cells, respectively ($n = 917$ and 888). Interestingly, the endothelial cell proliferation rate was significantly higher in nonpericyte-contacting endothelial cells cultured with Rho-altered pericytes (50.7 $\pm$ 3.1% for RhoDA, 53.9 $\pm$ 6.0% for RhoDN) compared with vector and uninfected controls (26.6 $\pm$ 2.7% and 30.0 $\pm$ 3.6%, respectively; $P < 0.05$ compared with either Rho-altered pericyte condition). Both of these effects were unique to Rho among small GTPases, as epitope tag-confirmed transfection of pericytes with dominant-active forms of the Ras, Rac, and Cdc42 small GTPases (see Materials and Methods) showed intact pericyte contact-mediated growth arrest by an average of 55.8 $\pm$ 2.2% (Ras 53.9 $\pm$ 5.0%, Rac 55.3 $\pm$ 4.4%, and Cdc42 58.2 $\pm$ 4.1%; $n = 479$, 533, and 478, respec-

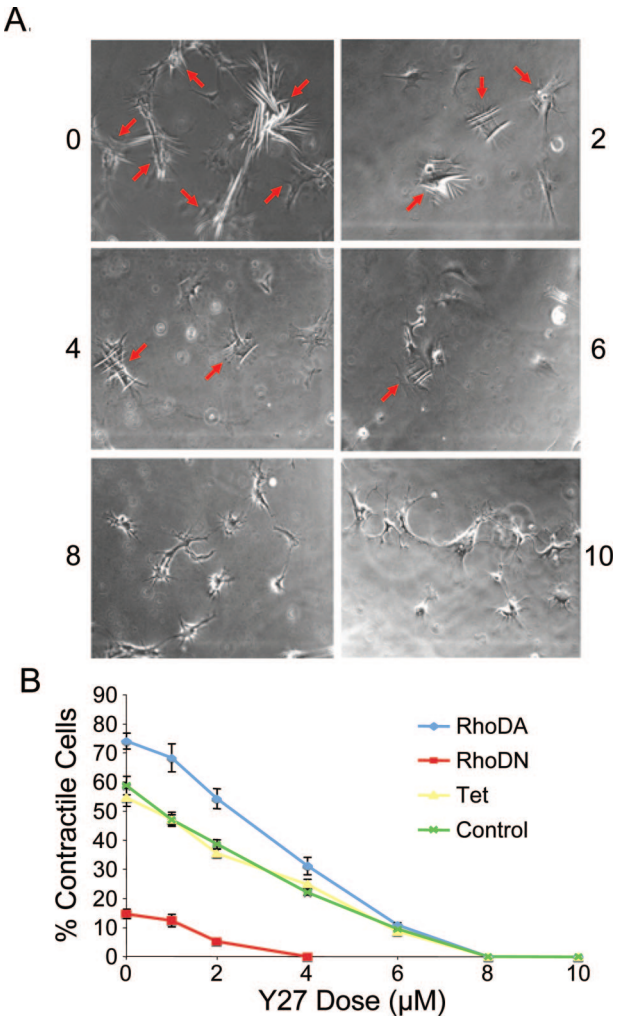

Figure 3. Wild-type and Rho GTPase-mutant pericyte contractility is dose dependently reversible by small molecule inhibition of Rho kinase. **A:** Bovine retinal pericytes were transduced with adenoviruses containing dominant-active Rho GTPase (RhoDA), dominant-negative Rho GTPase (RhoDN), transactivator alone (Tet), or mock-infected (control) as above. After 24 hours of culture, cells were trypsinized and replated onto plasma glow discharge-prepared, type I collagen-coated silicon substrates in media containing 0 to 10 μmol/L of the small molecule Rho kinase inhibitor Y-27632, and monitored by real-time phase-contrast imaging. Representative phase-contrast images of inhibitor-treated wild-type, mock-infected pericytes are provided as labeled, where **arrows** indicate substrate-wrinkling, actively contractile pericytes. Original magnifications, ×400. **B:** At 24 hours, contractility was assessed by the number of pericytes producing visible substrate wrinkling per each condition, expressed as mean percentages ± SE ($n > 100$ cells per condition, triplicate experiments.

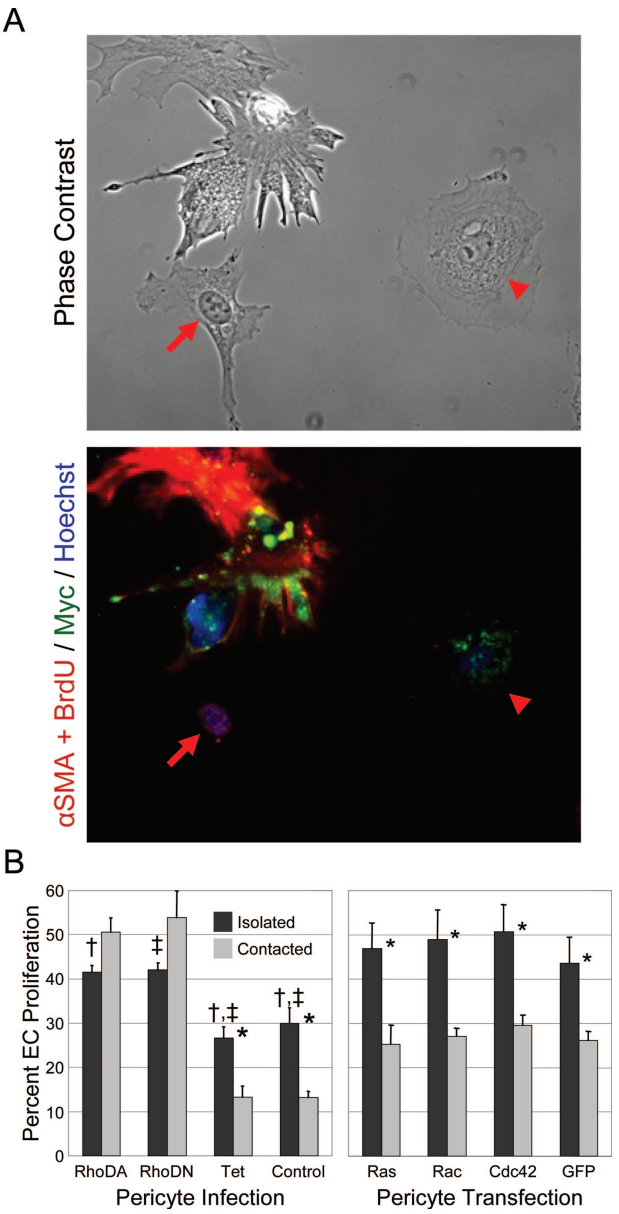

Figure 4. Adenoviral alteration of pericyte Rho (but not Ras, Rac1, or Cdc42) GTPase signaling impedes pericyte-mediated endothelial cell growth arrest. Bovine retinal pericytes were transduced with adenoviruses containing dominant-active Rho GTPase (RhoDA), dominant-negative Rho GTPase (RhoDN), transactivator alone (Tet), or mock-infected (control) as above and co-cultured with bovine retinal endothelial cells for 24 hours. BrdU was incorporated into the co-culture medium for the last 4 hours. **A:** Dominant-active Rho-infected pericyte co-cultures were then fixed and stained for the pericyte marker cytoplasmic α-smooth muscle actin and proliferative endothelial cell nuclear BrdU incorporation (αSMA + BrdU, red), the Myc epitope tag as a marker of infection for RhoDA (Myc, green), and nuclei by Hoechst (Hoechst, blue). Parallel phase images are provided. **Arrows:** A BrdU-positive, pericyte-contacting proliferating endothelial cell. **Arrowheads:** A lone BrdU-negative, quiescent endothelial cell. **B:** Adenoviral Rho GTPase-altered pericytes as above, as well as dominant-active Ras, Rac1, Cdc42, and GTP control-transfected pericytes, were co-cultured for 24 hours with bovine retinal endothelial cells as above. Co-cultures were then scored for nuclear BrdU-positive endothelial proliferation as a function of pericyte contact and GTPase status. Results are expressed as mean percentages ± SE ($n >$ 400 cells/condition, experiments in triplicate). $^{*}P < 0.05$ for differences between lone and pericyte-contacting endothelial cells with same GTPase status; $^{\dagger\ddagger}P < 0.05$ for differences between lone endothelial cell populations with different Rho GTPase status. Original magnifications, ×200.

tively), with no significant alteration in pericyte-mediated endothelial growth arrest compared with wild-type pericytes (60.0 ± 10.9%, $n = 515$). This parallels previous results indicating that Rho plays a unique role among the small GTPases in the modulation of isoactin array.[25]

Discussion

Here, we demonstrate that Rho GTPase-dependent signaling plays a pivotal role in multiple facets of pericyte biology; in particular, using both adenoviral infection and plasmid transfection of dominant-active and -negative Rho constructs, we directly demonstrate that Rho

GTPase pathway signaling through the isoactin network significantly alters Rho kinase-dependent cellular contractility. Moreover, using a novel *in vitro* assay of endothelial cell growth arrest, we demonstrate that pericyte regulation of microvascular endothelial proliferative status is impaired by either augmenting or diminishing Rho GTPase signaling. These effects are not reproduced by inhibition of signaling through other Ras-related small GTPases, including Ras, Rac, and Cdc42, expanding on previous work demonstrating that GTP-bound Rho, but not other Ras-related GTPases, specifically regulates pericyte contractility in an isoactin-specific manner.[25]

Mechanochemical Signaling in Pericytes

Our studies reveal key relationships between GTP-dependent signaling pathways and the microvascular pericyte cytoskeleton and its contractile effectors. The transforming growth factor (TGF)-β/SMAD signaling pathway has been implicated in retinal pericyte morphology and function,[33,34] as well as in pericyte-mediated retinal endothelial cell survival signaling.[35] Together, these data extend earlier observations implicating TGF-β as a key mediator of pericyte control of the endothelium.[36–38] Surprisingly, our data reveal that the proliferative index of nonpericyte-contacted endothelial cells in co-culture with both dominant-active and -negative Rho-infected pericytes is significantly increased above that of controls. This indicates the possibility that altered pericyte Rho expression may also alter secretion of a soluble mediator controlling endothelial proliferation. Therefore, we assayed by enzyme-linked immunosorbent assay levels of both total and activated TGF-β present in pericyte-conditioned culture media, as well as assessed for the presence of dominant-negative-acting soluble TGF-β receptor II into the media by Western blotting. Neither differences in levels of active or latent TGF-β nor the presence of soluble TGF-β receptor II was detected in dominant-active and -negative Rho-infected pericyte conditioned media (M.E.K., J.T. Durham, and I.M.H., unpublished observations). These findings are consistent with a recent study by Kondo and colleagues,[39] in which a TGF-β-1 blocking antibody only partially reversed the endothelial growth suppression caused by exposure to pericyte-conditioned media, whereas the effect was completely ameliorated by heat treatment of the conditioned media. In concert with our data, this suggests that there are probably additional mediators of the Rho-dependent control of pericyte-endothelial cellular dynamics yet to be revealed.

Pericyte Dysregulation in Angiogenesis

Results of our *in vitro* studies may lead to a more complete understanding of the role that pericytes play in orchestrating microvascular proliferative disorders, whether tumor-dependent,[40] diabetes-induced,[41] or associated with aging.[6] As we show here, pericyte signaling through a Rho GTPase-dependent pathway and its influence on endothelial cell growth arrest adds to a growing literature in support of pericytes' contributory role in regulating capillary endothelial cell function. Indeed, although it has been proposed that pericyte apoptosis or loss is associated with several angiogenic processes,[42] our findings strongly suggest that chronic alteration of pericyte Rho GTPase-dependent signaling may be sufficient for pathological angiogenesis to ensue. This underscores the importance of current work focusing on pericytes as novel drug targets in anti-angiogenic therapy.[18–21]

Pericytes in the Pathogenesis of Microvascular Tone Dysregulation

In addition to the role of disrupted endothelial control in vasoproliferative disease, microvascular signaling dysregulation is emerging as a causative factor in the pathogenesis of several nonproliferative vascular pathologies as well. Based on the initial understanding of Rho family GTPase signaling in the regulation of smooth muscle contractility,[43] a key role for Rho signaling through Rho kinase in the vascular system has recently been elucidated in both physiological maintenance[44] and pathophysiology.[24] In particular, arterial Rho GTP-dependent signal transduction may play a pivotal role in the pathogenesis of essential hypertension (as recently reviewed by Lee et al[23]). Regulation of vascular tone is thought to be governed by the balance between active calcium-dependent contractility[45] and nitric oxide-mediated relaxation[46,47] in smooth muscle. Emerging clinical data from human patients indicates that inhibition of the Rho kinase pathway via intravenous infusion of the Rho kinase inhibitor fasudil can correct aberrations in peripheral vascular tone present in heart failure,[48] coronary artery disease,[49] and even in cigarette smoking[50] with minimal tone alteration in normal controls. Our work suggests that an additional less well-explored component of the vascular pathologies accompanying essential hypertension may be linked to microvascular pericyte dysfunction or loss.

In the central nervous system, control of vascular tone is exquisitely sensitive to metabolic demands.[51,52] Functional imaging studies in the cerebellum have shown that *in vivo* moment-to-moment local control of demand-induced hyperemia in response to increased synaptic activity is dependent on neuronal nitric-oxide synthase.[53–56] Interestingly, inhibition of Rho kinase signaling by nitric oxide has been convincingly demonstrated in the regulation of extracranial arterial tone.[47] Further, *in situ* hybridization and immunohistochemical evidence indicates robust expression of Rho GTPase and Rho kinase in cerebellar tissue,[57] indicating that inhibition of Rho kinase signaling may be involved in cerebellar functional hyperemia. A similar potential role for Rho kinase has also been described in the hypertensive brainstem, including possible novel vasodilation-independent effects of Rho kinase activation in the regulation of the sympathetic nervous system.[58] These results in concert suggest that perturbations in Rho-mediated signaling may underlie both vasogenic and neurogenic mechanisms of hyper-

tension. Interestingly, early comparative studies on the cerebral microvasculature in hypertensive versus normotensive rats revealed increased pericyte investment of endothelial cells as well as loss of normal stress fiber distribution in pericytes associated with the hypertensive microvasculature both *in situ* and in cell culture.[59,60]

Parallel evidence from the retinal microvasculature supports the hypothesis that pericyte Rho kinase signaling may play a principle role in microvascular tone regulation. Careful dissection of smooth muscle contractile pathways in the hypertensive rat arterial system implicate activation of Rho kinase,[61,62] dynamically balanced by countervailing Rho kinase-mediated release of nitric oxide from adjacent endothelial cells.[63,64] In a pericyte-mediated parallel to this pathway in smooth muscle, calcium-dependent chloride channel activation mediates pericyte contractility in the retina,[65–67] in which nitric oxide has similarly been shown to counterbalance ligand-mediated contraction by promoting pericyte relaxation.[68,69] Interestingly, the retina is the most densely pericyte-invested vascular bed in the human body,[70] lacking the precapillary smooth muscle sphincters that play a regulatory role in other vascular beds.[71] Thus, pericyte Rho GTPase signaling through downstream Rho kinase effector pathways may play a microvascular bed-specific role parallel to that of smooth muscle regulation at the arteriolar level.

Our results indicate that pericyte contractility is Rho GTPase-dependent and Rho kinase-mediated. In concert with recent evidence that the pericyte is an autonomous regulator of capillary tone,[72] these data imply that the Rho kinase-mediated regulation of vascular tone in specific capillary beds may in fact originate locally at the level of the capillary-associated pericyte, with subsequent upstream conduction to proximal arteriole-associated smooth muscle cells. This intriguing possibility suggests that some elements of vascular tone dysregulation previously attributed solely to vascular smooth muscle may in fact be pericyte-mediated and that further investigation into the regulation of microvascular tone and endothelial cell regulation may elucidate novel capillary level signaling mechanisms involving multiple perivascular cell types.

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