Mechanisms of Nitric Oxide Interplay with Rho GTPase Family Members in Modulation of Actin Membrane Dynamics in Pericytes and Fibroblasts

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Migration of pericytes such as hepatic stellate cells is fundamentally important for diverse biological and pathological processes including tumor invasion and fibrosis. In prototypical migratory cells such as fibroblasts, the small GTPases Rac1 and RhoA govern the assembly of lamellipodia and stress fibers, respectively, cytoskeletal structures that are integral to the cell migration process. The gaseous signaling molecule nitric oxide (NO) influences growth factor chemotactic responses, although this occurs primarily in cell-type-specific ways and through cell biological effects that are poorly characterized. In this study, we use complementary molecular and cell biological approaches to delineate important roles for Rac1, RhoA, and NO in migration of the human hepatic stellate cell line LX2 and primary rat hepatic stellate cells. Both platelet-derived growth factor (PDGF) and Rac1 overexpression drove migration through formation of actin-positive filopodia spikes in LX2 as compared to the formation of lamellipodia in fibroblasts. NO inhibited PDGF- and Rac1-driven migration in LX2 by abrogating filopodia formation and inhibited migration of fibroblasts by attenuating lamellipodial protrusions. Additionally, RhoA conferred resistance to NO inhibition of migration and restored chemotactic responses to PDGF in the absence of functional Rac1 in LX2. In conclusion, these studies identify novel crosstalk between small GTPases, cytoskeletal structures, and NO in pericyte-specific pathways, providing counterbalances in the chemotactic responses to growth factors. (*Am J Pathol* 2005, 166:1861–1870)

Cellular locomotion requires dynamic but regulated actin remodeling to form membrane structures that facilitate cell extension.¹ These include lamellipodia, which are membrane protrusions that form the leading edge toward directed cell migration, and filopodia, which are thin, actin filament-structured spikes emanating from the plasma membrane.² Small GTP-binding proteins from the Rho family including RhoA (Rho), Rac1 (Rac), and Cdc42 in turn, closely regulate formation of actin-based structures.^{1,2} The canonical pathways by which Rho, Rac, and Cdc42 regulate actin remodeling are best characterized in fibroblasts, a prototypical migratory cell.^{3–5} In fibroblasts, Rho, Rac, and Cdc42 act in concert to regulate fibroblast motility with Rac required for lamellipodia protrusion, Rho promoting assembly of actin stress fibers, and Cdc42 contributing to filopodia and membrane ruffles.^{3–5} However, recent studies have highlighted further complexities to this paradigm including crosstalk, redundancy, antagonism, and cell-type specificity of these signaling pathways.⁵

Hepatic stellate cells (HSCs) are liver-specific pericytes.⁶ HSC locomotion is integral to a number of fundamental biological and disease processes in liver^{7,8} and recapitulates the importance of pericytes in other organs as well.⁹ For example, angiogenesis-based invasion of metastatic liver lesions requires migration of HSCs as does the process of hepatic scarring and fibrosis.^{10–12} Recent studies have expanded our understanding of HSC migration by elucidating the chemotactic role of platelet-derived growth factor (PDGF) on HSCs.^{13,14} However, unlike more prototypical migratory cells such as fibroblasts, the mechanisms in HSCs that govern small GTPase function, actin remodeling, and locomotion remain poorly defined in part because of methodological

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challenges in performing detailed molecular studies in HSCs.¹⁵

Nitric oxide (NO), derived from endothelial cells lying adjacent to HSCs, plays an integral role in the remodeling of vascular networks through paracrine signaling pathways some of which are mediated through its downstream targets, guanylate cyclase and protein kinase G (PKG).¹⁶ However, the sum influence and signaling effectors of NO signals on cell migration are complex and vary markedly between different cell types. Furthermore, the observed actions of NO on cell migration have not been translated into a rational understanding of how NO interdigitates with small GTPase function and actin-based membrane remodeling.

In this study, we demonstrate, using DNA microinjection of Rho family GTPases in combination with morphological and functional assays in LX2, a cell line derived from human HSCs,^{13,15,17–19} that 1) Rac promotes migration by causing formation of actin-positive, filopodia spikes; 2) NO and PKG inhibit PDGF- and Rac-driven migration by inhibiting filopodia formation; and 3) Rho confers resistance to NO inhibition of migration and also restores chemotactic responses to PDGF in the absence of functional Rac. Thus, these studies identify novel crosstalk between small GTPases, cytoskeletal structures, and NO signaling pathways that provides counterbalances in the process of cell motility and migration.

Materials and Methods

Cell Culture and Transfection

LX2 cells, rat HSCs, human skin fibroblasts (HSFs), NIH 3T3, and rat lung fibroblasts were used in this study. LX2 is a well-characterized cell line derived from human HSCs, which recapitulate many features of the activated HSC phenotype including expression of HSP 47 mRNA, MMP-2, and PDGF receptor β subunit.¹⁵ Unlike primary HSCs, LX2 are amenable to detailed molecular interventions and have been broadly used for this purpose.^{13,15,17–19} LX2 were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 1 mmol/L L-glutamine, and 100 IU/ml penicillin. HSCs were isolated from rat liver using collagenase digestion and density gradient centrifugation and cultured as we and others have previously described and studied between passages 1 to 3.^{20,21} HSF, NIH 3T3, and rat lung fibroblasts were grown in minimum Eagle's medium, supplemented with 10% fetal bovine serum, 1 mmol/L L-glutamine, and 100 IU/ml penicillin.

For adenoviral transduction, cells were washed with phosphate-buffered saline (PBS) and incubated with PBS/0.1% albumin containing 50 multiplicity of infection of adenoviral vector encoding endothelial nitric oxide synthase (AdNOS; kindly provided by Dr. W. Sessa, Yale University, New Haven, CT), PKG (AdPKG, kindly provided by Dr. K. Bloch, Massachusetts General Hospital, Boston, MA), or adenoviral vector encoding green fluorescent protein (AdGFP) for 60 minutes.^{17,22} The vector

solution was then aspirated, and after washing with PBS, complete culture medium was replenished and incubated for 24 hours. The transfection efficiency was confirmed by conventional epifluorescence microscopy and routinely close to 100%. cDNA encoding constitutive active and dominant-negative Rho (RhoQL and dnRho, respectively), constitutive active and dominant-negative Rac (RacQL and dnRac, respectively), and constitutive active and dominant-negative Cdc42 (Cdc42QL and dnCdc42, respectively), originally generated by Dr. J. Silvio Gutkind (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD) by site-directed mutagenesis, were kindly provided by Dan Billadeau (Mayo Clinic, Rochester, MN) and Federico Kalinec (University of California at Los Angeles, Los Angeles, CA).²³ Transient transfections were performed in LX2 using a standard calcium phosphate method with efficiency approximating 50%. Co-transfection of adenoviral vectors and plasmid vectors, or multiple plasmid vectors, were performed in some studies as indicated.

Migration Assay

Cellular chemotaxis was measured using modifications of a Boyden transwell migration assay, in which transwells were fitted with polyvinylpyrrolidone-free polycarbonate membranes (13-mm diameter, 8- μ m pore size; Neuro Probes, Inc., Gaithersburg, MD). Polycarbonate filters were precoated with 50 μ g/ml of human type I collagen for 30 minutes at 37°C. The bottom wells of the chamber were filled with 26 μ l of serum-free media containing 10 ng/ml of PDGF or vehicle. Wells were covered with the coated membrane sheet, and 20,000 serum-starved cells were added into the upper chamber of each well. Test agents were added alone, or in combination, to the upper chamber of transwells (50 μ l) and included the NO donor, sodium nitroprusside (SNP; 50 to 500 μ mol/L), and an inhibitor of guanylate cyclase, ODQ (100 μ mol/L). The chamber was incubated for 4 hours (37°C, 5% CO₂), after which cells from the upper surface of membranes were completely removed with gentle swabbing. The remaining migrated cells on the lower surface of membranes were fixed and stained using Hema3 stain according to the manufacturer's recommendations (Biochemical Sciences, Inc., Swedesboro, NJ). Membranes were then rinsed with distilled water and mounted onto glass slides. Cellular migration was determined by counting the number of stained cells on membranes in five randomly selected, nonoverlapping high-power fields. Each experiment was repeated three to five separate times with three to five replicate wells for each test condition.

Microscopic Analysis of Plasma Membrane Actin Structures

For phalloidin staining, cells were cultured on tissue culture slides, fixed in 4% paraformaldehyde in phosphate-buffered saline for 30 minutes at 4°C, and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline

for 1 minute at room temperature. Filamentous actin was stained with tetramethyl-rhodamine isothiocyanate (TRITC)-phalloidin in phosphate-buffered saline (1 $\mu\text{mol/L}$) for 20 minutes at room temperature. After mounting, cells were visualized by confocal laser-scanning microscopy. In some studies, cells were scored as positive or negative for lamellipodia or filopodial spikes. Lamellipodia-positive cells were defined as having at least one broad flat actin-positive protrusion, which was aligned parallel to the cell membrane, whereas filopodia-positive cells were defined as having more than 10 thin, actin-positive processes projecting beyond the cellular edge of the plasma membrane.²⁴ Quantitative analyses were performed in a blinded manner from 200 individual cells per each group and from three independent experimental preparations.

For live cell imaging, cells were plated and cultured on 32-mm coverslips and mounted in fresh medium in the 37°C stable temperature control chamber (POC-R; Zeiss, Oberkochen, Germany).²⁵ The chamber was placed on the stage of a microscope (Pasqual LSM 5; Zeiss) and covered. PDGF (10 ng/ml) was added to the chamber and phase contrast images were captured every minute for 25 minutes using a $\times 63$ C-apochromat lens. To measure the area of lamellipodia protrusion, cell morphology was traced using Adobe Photoshop (Adobe Systems, Mountain View, CA) from images that were captured every 5 minutes from live cells. The area of lamellipodia protrusion and the starting area of each cell were quantitated using NIH Image, and the ratio of these two values was calculated and expressed as a percentage per cell for each of the conditions with 10 to 15 cells analyzed per experimental group.

DNA Microinjection

For microinjection experiments, cells (10^4) were plated and cultured on 55- μm^2 CELLocate coverslips (Eppendorf Scientific, Inc., Hamburg, Germany). Optimal injections were obtained with sterile femtotips II (diameter of opening of tip, $0.5 \mu\text{m} \pm 0.2 \mu\text{m}$; Eppendorf Scientific, Inc.). RhoQL, RacQL, or empty plasmid DNA (10 $\mu\text{g/ml}$) were dialyzed in microinjection buffer (10 mmol/L KH_2PO_4 , pH 7.2, and 75 mmol/L KCl) and Alexa Fluor 488 (hydrazide sodium salt, 200 mmol/L KCl; Molecular Probes, Eugene, OR) was added to all microinjection solutions to enable identification of injected cells. Cells were pressure injected using an Eppendorf InjectMan NI2 and FemtoJet 5247 on a Zeiss Axiovert inverted microscope, with an optimized program adjusted for injection pressure (P_i , 140 hPa; 1 hPa = 0.0145 PSI), compression pressure (P_c , 40 hPa), and time (T , 0.4 second).²³ Images were obtained using an intensified charge-coupled device camera attached to a Zeiss Axiovert 35 microscope (Carl Zeiss, Inc.). After injection, cells were allowed to recover 4 to 6 hours at 37°C in a 5% CO_2 incubator and then treated with vehicle or 500 $\mu\text{mol/L}$ SNP. Twenty-four hours after injection, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline and stained for actin with TRITC-phalloidin in phos-

phate-buffered saline (1 $\mu\text{mol/L}$). Quantitation of filopodia was performed by counting filopodial spikes from captured images of injected cells ($n = 5$ cells per experimental group).

Rac and Rho Activation Assays and Western Blot

Rac and Rho activation assays were performed as described previously.²⁶ In brief, glutathione *S*-transferase (GST)-p21-binding domain (GST-PBD) and Rhotekin Rho binding domain (GST-TRBD) fusion constructs, kindly provided by Dr. D. Mukhopadhyay, Mayo Clinic, Rochester, MN were transformed into BL21 (DE3) and induced with IPTG (1 mmol/L) overnight. Bacterial suspensions were prepared, aliquotted, and frozen at -80°C . To prepare the GST-PBD and GST-TRBD beads, each aliquot of frozen bacteria was resuspended in 2 ml of cold PBS, and then 20 μl of 1 mol/L dithiothreitol, 20 μl of 0.2 mol/L phenylmethyl sulfonyl fluoride, and 40 μl of 50 mg/ml lysozyme were added and incubated on ice for an additional 30 minutes. Ten percent Triton X-100 (225 μl), 22.5 μl of 1 mol/L MgCl_2 , and 22.5 μl of 2000 kU/ml DNase were then added, and the samples were incubated on ice for another 30 minutes. The supernatant was collected and incubated with 100 μl of glutathione-coupled Sepharose 4B beads (Amersham Biosciences) at 4°C for 45 minutes. The beads were then washed (PBS with 10 mmol/L dithiothreitol and 1% Triton X-100) and resuspended in the same buffer to generate a 50% bead slurry. For binding experiments, Rac, Rho, or mock-transfected cells were lysed with a buffer (150 mmol/L NaCl, 0.8 mmol/L MgCl_2 , 5 mmol/L EGTA, 1% IGEPAL, 50 mmol/L HEPES, pH 7.5, 1 mmol/L phenylmethyl sulfonyl fluoride, 10 $\mu\text{g/ml}$ leupeptin, and 10 $\mu\text{g/ml}$ aprotinin). In some experiments cells were pretreated with 500 $\mu\text{mol/L}$ SNP for 1 hour before lysis. The supernatant was isolated and incubated with 50 μl of GST-PBD or GST-TRBD beads at 4°C for 45 minutes. Bound proteins were washed with a buffer (50 mmol/L Tris-HCl, pH 7.2, 1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L MgCl_2 , 1 mmol/L phenylmethyl sulfonyl fluoride, 10 $\mu\text{g/ml}$ leupeptin, and 10 $\mu\text{g/ml}$ aprotinin), eluted, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed by Western blot with pAb specifically recognizing Rac or Rho (Santa Cruz Biotechnologies, Santa Cruz, CA). For positive control, cell lysates were incubated for 15 minutes at 30°C in the presence of 10 mmol/L EDTA and 100 $\mu\text{mol/L}$ GTP γS . Western blot from cell lysates was also performed using eNOS mAb (Transduction Laboratories, Lexington, KY).

Statistical Analysis

Data are presented as mean $\pm$ SE. Data were analyzed using paired and unpaired Student's *t*-tests as appropriate.

Figure 1. PDGF-dependent migration of LX2 is dependent on Rac. **A:** Boyden migration assays were used to study chemotactic responses to PDGF (10 ng/ml) in HSF or in LX2 transfected with plasmids encoding RacQL, RhoQL, Cdc42QL, or empty vector. Both fibroblasts and LX2 evidenced chemotactic responses to PDGF. In LX2, although RhoQL and Cdc42QL transfection did not influence basal- or PDGF-induced migration, RacQL transfection markedly increased basal migration and migration in response to PDGF compared to cells transfected with empty vector ($^*P < 0.05$, PDGF versus vehicle in fibroblasts; $^*P < 0.05$, PDGF versus respective vehicle; $^{**}P < 0.05$, RacQL vehicle versus empty vector vehicle; $^{***}P < 0.05$, RacQL PDGF versus empty vector PDGF; $n = 4$ independent experiments with three to five replicates of each group per experiment). **B:** LX2 were transduced with plasmid-encoding empty vector, dnRac, dnRho, or dnCdc42, and chemotactic responses to PDGF were examined. PDGF-induced migration responses in cells transduced with dnRac were attenuated. LX2 transduced with dnCdc42 or dnRho evidenced chemotactic responses similar to cells transduced with empty vector ($^*P < 0.05$ versus respective vehicle).

Results

Regulation of PDGF-Dependent LX2 Migration by Rac

The small GTPases, Rac, Rho, and Cdc42 mediate signaling pathways, which regulate the formation of distinct actin-based structures essential to cell motility and chemotactic responses. Therefore, we examined the influence of Rho family small GTPases on LX2 migration by transfecting cells with the constitutive active constructs RacQL, RhoQL, Cdc42QL, or the dominant-negative constructs dnRac, dnRho, or dnCdc42, and assessing migration in response to the prototypical chemotactic growth factor, PDGF, using a modified Boyden chamber assay. As seen in Figure 1A, both LX2 and a prototypical migratory fibroblast cell, HSF, evidenced chemotactic responses to PDGF, although the magnitude of effect was sometimes quantitatively greater in fibroblasts. In

transfection experiments performed in LX2, although neither RhoQL nor Cdc42QL influenced basal or PDGF-induced migration, RacQL significantly increased basal migration and migration in response to PDGF compared to cells transfected with empty vector. As seen in Figure 1B, in experiments using the dominant-negative constructs in LX2, dnRac inhibited PDGF-induced migration whereas neither dnRho nor dnCdc42 influenced PDGF-induced migration. These studies highlight the important role of Rac in PDGF-induced migration of LX2.

Rac and PDGF Promote Filopodia Formation in HSCs but Not in Fibroblasts

In fibroblasts, PDGF initiates a PI-3 kinase-dependent pathway that activates Rac and promotes lamellipodia.⁵ However, the influence of PDGF on actin membrane structures in HSCs is unexplored. Therefore, we performed microscopic analysis of actin membrane structures in response to PDGF to compare and contrast events in fibroblasts and LX2. HSF in serum-free medium have sporadic thin protrusions (Figure 2A, left). PDGF promotes formation of broad plasma membrane extensions parallel to the substrate, characteristic of lamellipodia (Figure 2A, right). A larger percentage of fibroblasts evidenced protrusions in response to PDGF in a time-dependent manner (Figure 2A, far right graph). However, PDGF had a surprisingly different effect in LX2. Similar to fibroblasts, LX2 were relatively devoid of lamellipodia in serum-free control conditions (Figure 2B, left). However, PDGF stimulated the proliferation of spike-like filopodia structures rather than lamellipodia (Figure 2B, right). This occurred in a time-dependent manner as evidenced in the graph (Figure 2B, far right graph) with the majority of cells evidencing numerous filopodial structures after PDGF stimulation. Prominent filopodial structures were also observed in primary rat HSCs in response to PDGF (Figure 2C; middle panel with arrow pointing to higher magnification image).

As RacQL promoted basal- and PDGF-induced migration in LX2, we next examined actin membrane structures in cells microinjected with RacQL DNA. Cells were microinjected with RacQL, RhoQL, or empty plasmid (10 μ g/ml), and co-injected with Alexa Fluor 488 to identify microinjected cells. Twenty-four hours later, cells were fixed and stained with phalloidin. Although cells injected with empty vector evidenced no substantive changes in actin staining (Figure 2D, left), significant morphological changes in HSCs were observed after 24 hours of RacQL nuclear microinjection. RacQL DNA-injected cells developed numerous actin-positive, extended filopodia spikes reminiscent of that seen in response to PDGF (Figure 2D, right). This effect was observed in each of the five cells that were studied after microinjection and the graph (far right) depicts computation of the significantly increased number of filopodial structures per cell in LX2 microinjected with RacQL as compared to empty plasmid or RhoQL. In contrast, cells injected with RhoQL appeared to evidence an increase in stress fiber formation compared to control cells (Figure 2D, middle), although the

Figure 2. PDGF and Rac promote formation of filopodia spikes in LX2 but lamellipodia in fibroblasts. Microscopic analyses were performed in fibroblasts and LX2. Cells were stained with TRITC-phalloidin at indicated times after PDGF or DNA microinjection. **A:** Fibroblasts in serum-free medium are elongated and have small sporadically located lamellipodia protrusions (**left**). PDGF promoted broad plasma membrane extensions characteristic of lamellipodia (**right, arrow**). Quantification of the percentage of cells with lamellipodia or filopodial spikes was performed in a blinded manner. PDGF increased the number of cells that had protrusions in a time-dependent manner (**far right**; $*P < 0.05$ versus time 0; $n = 200$ cells from three independent experiments). **B:** LX2 in serum-free media were devoid of lamellipodia and filopodial spikes (**left**). Contrary to fibroblasts, PDGF promoted the formation of spike-like filopodia structures in LX2 (**right, arrows**), and quantitative analysis demonstrated that this effect occurred in a time-dependent manner (**far right**; $*P < 0.05$ versus time 0; $n = 200$ cells from three independent experiments). **C:** Primary rat HSCs were deficient in lamellipodia and filopodial spikes in serum-free conditions (**left**). Filopodial spikes became more prominent in response to PDGF (**right**; higher magnification image is depicted in the **box** to the **far right** indicated by the **arrow**). **D:** LX2 were microinjected with DNA encoding RacQL, RhoQL, or empty plasmid DNA and co-injected with Alexa Fluor 488 to identify injected cells. Phase contrast images of injected cells are shown at the **top**. Twenty-four hours after injection, cells were stained with TRITC-phalloidin and observed with confocal laser-scanning microscopy. No morphological changes were observed after nuclear injection of empty vector (**left**). Cells injected with RhoQL evidenced some increase in stress fiber formations (**middle**), although the effect was not so prominent. Cells injected with RacQL developed prominent actin-positive, extended filopodia spikes (**right**; higher magnification image is depicted in the **boxed inset**). Micrographs are representative from five independent experiments. The graph (**far right**) depicts computation of the significantly increased number of filopodial structures per cell in LX2 microinjected with RacQL as compared to empty plasmid or RhoQL ($*P < 0.05$; RacQL versus empty vector).

cellular effects were not as prominent as that observed in cells injected with Rac.

NO Inhibits Rac and PDGF-Induced Cell Migration

The stimulatory effect of RacQL overexpression on PDGF-induced HSC chemotaxis and on filopodia forma-

Figure 3. NO inhibits PDGF- and Rac1-dependent migration. Migration assays were performed by Boyden chamber assay ($n = 3$ independent experiments with three to five replicates of each group per experiment). **A, left:** SNP (0 to 500 $\mu\text{mol/L}$) inhibited PDGF-dependent chemotaxis in a concentration-dependent manner in LX2 cells and HSFs ($*P < 0.05$ versus LX2 vehicle; $*P < 0.05$ versus fibroblast vehicle). **Right:** SNP (500 $\mu\text{mol/L}$) also reduced PDGF-dependent chemotaxis to levels even below basal migration in rat HSCs, and other representative fibroblast cells including rat lung fibroblasts and NIH 3T3 cells ($*P < 0.05$ versus vehicle in each cell type). **B:** The effect of AdNOS gene transfer on PDGF-dependent migration of LX2 and fibroblasts was examined by Boyden chamber assay. Migration in response to PDGF was attenuated in both LX2 and fibroblasts transduced with AdNOS, as compared to cells transduced with AdGFP ($*P < 0.05$ versus AdGFP). **C:** The effect of NO on Rac-induced LX2 migration was examined by Boyden chamber assay. Both SNP as well as AdNOS gene transfer markedly attenuated PDGF-induced migration in LX2 transfected with RacQL ($*P < 0.05$ versus LX2 vehicle). The **boxed** Western blot analyses confirm overexpression of RacQL and NOS as evidenced by increased active Rac and increased eNOS protein levels in cell lysates from respectively transfected LX2.

tion, in conjunction with the key role of NO on vascular remodeling and cell migration, prompted us to next examine the influence of NO on this chemotactic pathway in HSCs. Although HSCs do not basally express NOS, they are influenced by NO signals from adjacent endothelial cells and do express NOS under specific circumstances.²⁷ Influence of NO signals was studied by complementary approaches, including NOS gene transfer to mimic endogenous intracellularly produced NO and the NO donor SNP to resemble paracrine-derived NO signals. First, LX2 and HSF were treated with the NO donor SNP at varying concentrations at the time of initiation of the migration assay. In both cell types, SNP inhibited PDGF-induced chemotaxis in a concentration-dependent manner with almost complete inhibition of PDGF-induced migration observed at 500 $\mu\text{mol/L}$ SNP (Figure 3A, left). Qualitatively, similar results demonstrating SNP inhibition of PDGF-induced chemotaxis were obtained in primary isolates of rat HSCs as well as two other representative

fibroblast cell types including rat lung fibroblasts and NIH 3T3 cells (Figure 3A, right). Next, experiments were performed after transduction of cells with AdNOS or AdGFP. Chemotactic response to PDGF was attenuated in both LX2 and HSFs that were transduced with AdNOS, as compared to PDGF chemotaxis in cells transduced with AdGFP, although the magnitude of effect of NO inhibition in these experiments appeared larger in fibroblasts (Figure 3B). The detected inhibition of PDGF-induced chemotaxis in response to NO was not secondary to NO-induced apoptosis as evidenced by a lack of increase in DNA fragmentation in cells transduced with AdNOS as compared to cells transduced with AdGFP (percent apoptotic cells: AdGFP, $2.7 \pm 0.3\%$; AdNOS, $3.0 \pm 0.2\%$; NS).

We next examined the effect of NO on Rac-induced LX2 migration. As seen in Figure 3C, both SNP as well as AdNOS gene transfer markedly attenuated PDGF-induced migration in cells overexpressing RacQL, reducing chemotactic responses almost to that level observed in control cells. These studies establish an inhibitory effect of NO signaling on PDGF- and Rac-induced LX2 migration. The biological effectors of some NO signaling responses include guanylate cyclase, which generates cGMP in response to NO stimulation. This led us to examine how modulation of the guanylate cyclase pathway affects LX2 migration. As seen in Figure 4A, the guanylate cyclase inhibitor ODQ did not rescue fibroblasts from the inhibitory effect of NO signals derived from AdNOS on migration responses to PDGF nor did ODQ abrogate the inhibitory effects of NO signals derived from SNP on migration responses to PDGF (Figure 4B). The lack of effect of ODQ was corroborated by direct measurements of cellular cGMP levels in LX2 under basal- and SNP-stimulated conditions, which demonstrated that cGMP levels were barely detectable in LX2 cells (data not shown). These results in LX2 are consistent with previous data by Failli and colleagues²⁸ demonstrating that guanylate cyclase expression is down-regulated in highly activated HSCs. We next examined if adenoviral gene delivery of a kinase downstream from NO and guanylate cyclase, PKG, could reconstitute inhibition of PDGF-induced migration in LX2. Owing to the lack of cGMP detected in LX2, these experiments were all performed in the presence of the exogenous PKG substrate, 8-Br-cGMP. As seen in Figure 4C, migration in response to PDGF was markedly inhibited in HSCs transduced with AdPKG as compared to cells transduced with AdGFP reminiscent of that observed with AdNOS gene transfer. These studies indicate that in LX2, NO inhibits PDGF-induced migration independent of guanylate cyclase but that PKG can mimic the inhibitory effects of NOS overexpression on migration responses.

NO Inhibits Rac-Dependent Filopodia Formation in LX2

We next sought to examine the mechanism by which NO inhibits migration responses induced by PDGF in HSCs and contrast this to fibroblasts. First, HSFs were trans-

Figure 4. NO inhibits Rac- and PDGF-induced migration independent of guanylate cyclase. Migration assays were performed by Boyden chamber assay ($n = 3$ independent experiments with three to five replicates of each group per experiment). **A:** In fibroblasts, the guanylate cyclase inhibitor, ODQ, did not rescue cells from the inhibitory effect of NOS-derived NO generation ($*P < 0.05$ versus vehicle). **B:** ODQ did not reverse SNP-mediated inhibition of PDGF-induced LX2 migration. ($*P < 0.05$ versus vehicle). **C:** LX2 were transduced with AdPKG or AdGFP, incubated with the PKG substrate, 8-Br-cGMP, and migration responses to PDGF were examined. In cells transduced with AdPKG, migration in response to PDGF was attenuated as compared to cells transduced with AdGFP ($*P < 0.05$ versus vehicle).

ected with AdGFP or AdNOS and 24 hours later were mounted on a microscope for real-time evaluation of dynamic membrane changes in response to PDGF and NO signals in live cells. As seen in Figure 5A, in fibroblasts transfected with AdGFP, PDGF increased lamellipodial protrusion area in a time-dependent manner. However, protrusion extension in response to PDGF was attenuated in fibroblasts transduced with AdNOS by 50% compared to cells that were transduced with AdGFP. The attenuation in PDGF-induced lamellipodial protrusion in response to NO signals is also depicted in the representative micrographs of phalloidin-stained cells obtained after 30 minutes of observation. Further complimentary quantitative analyses demonstrated that AdNOS transduction was also associated with a corroborative reduction in the number of cells evidencing lamellipodial protrusions in response to PDGF treatment (data not shown). As PDGF and RacQL induced the proliferation of filopodial spike structures rather than lamellipodia in LX2, we next examined the influence of NO on this process more directly by performing RacQL DNA microinjection studies. In LX2

Figure 5. NO inhibits Rac-dependent filopodia structures in LX2 but lamellipodia in fibroblasts. **A:** Motility analyses were performed using AdGFP- or AdNOS-transduced fibroblasts. For live-cell imaging, cells were cultured on 32-mm coverslips and mounted in fresh medium in a 37°C stable temperature control chamber. Vehicle or 10 ng/ml of PDGF was added to the chamber and phase contrast images were captured every minute for 25 minutes. Every 5 minutes, the area of lamellipodia protrusion and the starting area of each cell were quantitated. Quantitation of cell area was performed in a blind manner from 10 to 15 individual cells from five independent experimental preparations. The ratio of the area of lamellipodia protrusion to the initial area of the cell was calculated, and the results are presented as a percentage of cell area change throughout time. Micrographs depict representative phalloidin-stained cells from each group at end of observation period (arrows indicate lamellipodial protrusions). Fibroblasts transduced with AdNOS evidenced attenuated protrusion area in response to PDGF compared with cells transduced with AdGFP in response to PDGF. Negligible changes in protrusion area were observed in cells in the absence of PDGF. **B:** LX2 were microinjected with RacQL DNA and co-injected with Alexa Fluor 488 to identify injected cells. **Left:** Phase contrast images depict injected cells. After injection, cells were allowed to recover 4 to 6 hours at 37°C in a 5% CO₂ incubator and then treated with vehicle or 500 μ M SNP. Twenty-four hours after injection, cells were stained with TRITC-phalloidin and observed by confocal laser-scanning microscopy. An abundance of filopodial spikes were detected in cells injected with RacQL (**top**; higher magnification image is depicted in the **boxed inset**); however in cells injected with RacQL that were also incubated with SNP, filopodial spike formation was markedly reduced (**bottom**; higher magnification image is depicted in the **boxed inset**). Data shown are representative of five independent experiments. The graph (**far right**) depicts computation of the reduction in number of cellular filopodial structures in LX2 microinjected with RacQL and incubated with SNP as compared to cells microinjected with RacQL ($P < 0.05$; RacQL + SNP versus RacQL alone). **C:** LX2 transfected with RacQL or RhoQL were incubated with SNP or vehicle for 1 hour, followed by measurement of active Rac and Rho, respectively, via a pull-down experiment, as described in Materials and Methods. **Left:** Western blot using Rac pAb demonstrates that Rac activity was increased in cells transduced with RacQL however SNP did not reduce levels of active Rac ($n = 3$ independent experiments). Densitometric analysis from three independent experiments evidences increased Rac activity in response to RacQL transfection but no inhibition by SNP is depicted below the blot. **Right:** Western blot using Rho pAb demonstrates that Rho activity was increased in cells transduced with RhoQL however SNP did not reduce levels of active Rho ($n = 3$ independent experiments). Densitometric analysis from three independent experiments evidences increased Rho activity in response to RhoQL transfection but no inhibition by SNP is depicted below the blot.

injected with RacQL DNA, an abundance of filopodial spikes was detected on phalloidin staining of cells (Figure 5B, top). However, in LX2 injected with RacQL DNA that were treated with SNP, actin-positive filopodia spike formation was dramatically reduced (Figure 5B, bottom). This effect was observed in each of five cells that were studied and quantitation of the reduction in number of filopodia per cell in LX2 injected with RacQL and incubated with SNP as compared to cells injected with RacQL, is shown in the graph (Figure 5B, far right). Because signals downstream from Rac are responsible for filopodial spikes in LX2 and are blocked by NO, we hypothesized that NO may inhibit signals either at or downstream from Rac. Therefore, we next examined whether NO directly inhibits Rac activation by pull-down assay using GST-PBD fusion protein that binds selectively to GTP-bound active Rac. LX2 transfected with empty vector or RacQL were treated with SNP or vehicle. As seen in Figure 5C (left), these analyses demonstrated Rac activation in cells overexpressing RacQL compared to cells treated with empty vector. However, direct inhibition of Rac activity by SNP was not detected. Similar results were obtained in cells transfected with AdNOS used to generate NO signals in place of SNP (data not shown). A parallel protocol was used to examine the influence of SNP on Rho activity except that GST-PBD fusion protein was substituted with GST-TRBD, which binds to GTP-bound Rho. As seen in Figure 5C (right), although Rho overexpression was associated with increased Rho activity, SNP inhibition of Rho was not detected. Taken together, these studies indicate that although the inhibitory effect of NO signals in fibroblast migration is due to effects on lamellipodia, inhibitory effects of NO in LX2 occur through effects on filopodia formation through signals residing downstream from Rac but not through direct biochemical inhibition of Rac or Rho catalytic activity.

Rho Promotes HSC Migration in the Presence of NO and Absence of Rac

In fibroblasts, Rho is frequently activated downstream from Rac,⁴ however the interplay of Rho and Rac in HSCs is less understood. Because NO signals inhibited Rac-dependent actin structures, we next examined the influence of RhoQL overexpression on NO inhibition of PDGF-induced LX2 chemotaxis. In contrast to the SNP inhibition of migration that was observed in HSCs transfected with RacQL, HSCs transduced with RhoQL were completely protected from SNP inhibition of PDGF-induced migration (Figure 6A). Experiments were also performed after co-transfection of HSCs with both RhoQL and AdNOS to assess the influence of Rho activation on locally derived NO inhibitory signals in HSCs. Akin to that observed in the aforementioned experiments using the NO donor SNP, RhoQL rescued cells from the migration inhibition phenotype conferred by AdNOS (Figure 6A).

We next examined if RhoQL could rescue LX2 from inhibition of PDGF-induced migration conferred by dnRac by co-transfecting cells with both dnRac and RhoQL. Although

Figure 6. Rho promotes HSC migration in the presence of NO and absence of Rac. Migration assays were performed by Boyden chamber assay ($n = 3$ independent experiments with three to five replicates of each group per experiment). **A:** LX2 were transfected with plasmid encoding RhoQL and in some experiments were co-transfected with AdNOS, and chemotactic responses to PDGF were examined. RhoQL transfection rescued cells from the migration inhibition phenotype conferred by SNP and by AdNOS gene transfer ($*P < 0.05$ versus empty vector vehicle cells). The **boxed** Western blot analyses confirm overexpression of RhoQL and NOS as evidenced by increased active Rho and eNOS protein in cell lysates from respectively transfected LX2. **B:** LX2 were transfected with empty vector, dnRac, RhoQL, or both dnRac and RhoQL and chemotactic responses to PDGF were examined. Although dnRac inhibited PDGF-induced migration, co-transfection of RhoQL and dnRac, reconstituted PDGF-induced migration despite the presence of dnRac ($*P < 0.05$; PDGF versus respective vehicle).

RhoQL did not influence PDGF-induced migration, co-transfection of RhoQL with dnRac rescued cells from the impaired PDGF-induced migration observed with transfection of dnRac alone (Figure 6B). These studies indicate that Rho, can reconstitute PDGF-induced chemotaxis in the absence of functional Rac.

Discussion

Hepatic vascular structure is unique from other organ capillary beds in part because of a highly developed and essential pericyte structure that envelops the adjacent abluminal liver endothelial cell layer.²⁹ These liver pericytes, termed HSCs, also perform hepatic functions frequently ascribed to fibroblast cells including deposition of collagen and migration in response to wounding and angiogenic stimuli such as those that occur within the tumor metastasis process.^{7,8,10–12} Therapeutic tumor anti-angiogenesis has generally focused on the endothelial cell as a target.^{30,31} However, recent studies have

identified the liver pericyte as a novel target for anti-angiogenic therapies particularly applicable to primary and metastatic liver tumors, making the biology of these cells of particular interest.^{10,11,32} Because migration is a key step in the angiogenic cascade, the present studies were aimed at identifying novel insights into pericyte-specific motility and migration with an emphasis on the interplay of NO with Rho family small GTPases and actin-based membrane structures. Using complementary cell and molecular biological approaches in the liver pericyte cell line LX2 as well as in primary rat HSCs, we make several novel observations. Firstly, Rac drives migration through the formation of spike-like filopodia. Additionally, NO inhibits Rac- and PDGF-driven migration by preventing the formation of filopodia. An important role for Rho is also unmasked in the presence of NO signals, which are counteracted by Rho, and in the absence of functional Rac, in which case Rho can restore PDGF-induced migration.

Cell protrusion, and subsequent crawling, is driven in part by polymerization of actin within lamellipodia and filopodia, organelles that are regulated by signaling pathways driven through Rho, Rac, and Cdc42.¹ Small GTPase regulation of actin cytoskeleton in response to chemotactic growth factors is best characterized in fibroblasts. Previous studies have demonstrated that in fibroblasts, Cdc42 promotes filopodia; Rho, thought to be situated downstream from Rac, promotes stress fiber formation; whereas Rac drives lamellipodia formation.^{3,4} However, this hierarchical paradigm is less applicable in other cell types. Furthermore, signaling redundancy and cross-talk in these pathways has allowed for looser and sometimes contrary interpretations of these paradigms.⁵ Overall, the interplay of these pathways has not been studied in pericytes such as those residing in liver, in which migration is essential to a number of pathophysiological conditions including cirrhosis and tumor angiogenesis. In our studies, although Rac-induced lamellipodia drive migration in prototypical motile cells such as fibroblasts,^{3,4} LX2 were relatively devoid of lamellipodia, and Rac promotes filopodia spikes rather than lamellipodia protrusions as directly evidenced by microinjection of DNA encoding RacQL. An important role for these Rac-dependent spikes in the migration of LX2 is evidenced by the observation that inhibition of filopodia formation by NO signals also inhibits migration responses. A recent study also supports a primary role for Rac-induced filopodia in border cell migration during *Drosophila* oogenesis.³³ What are the reasons for differing interplay of small GTPases and actin membrane structures in different cell types? Interestingly, Rac signals link to filopodia formation in many central nervous system cell types also.³⁴ As HSCs as well as pericytes in other organs maintain several phenotypic similarities to central nervous system type cells, as evidenced by expression of the glial precursor proteoglycan, NG2, neural cell adhesion molecule, N-CAM, and the neuronally enriched, small GTPase RhoN, parallels in cellular origin of these cell types may be important in the phenotypic differences detected in these cells compared to fibroblasts and other epithelial type cells.^{35–38} Therefore, the specialized nature of cer-

tain cell types may result in such adaptive responses whereby Rac promotes migration through filopodia formation. It is also possible that the activation state of HSCs may also influence the interplay between small GTPases and actin dynamics as LX2 mimics the phenotype of highly activated HSCs.¹³ Although Cdc42 and Rho have little noticeable effect on basal migration or PDGF chemotaxis, the prokinetic actions conferred by Rho are evidenced under conditions that counteract PDGF chemotaxis including Rac loss-of-function or augmentation of NO signals. However, biochemical inhibition of Rac or Rho GTPase activity was not detected in the presence of NO suggesting that inhibitory actions of NO on these pathways may occur at the level of a regulatory guanine exchange factor or GTPase activating protein, rather than directly on the catalytic activity of Rac/Rho themselves.⁵

Anti-angiogenic therapies for cancer have focused in large part on the endothelial cell as a target. Owing in part to its stimulatory effect on endothelial cell permeability and migration,^{26,39} NO has been implicated in the process of tumor angiogenesis as well.⁴⁰ Indeed, NO is an essential downstream signal for vascular endothelial growth factor-dependent endothelial cell activation.^{26,39} However, tumor microvasculature in metastatic and primary liver cancers is comprised of not only endothelial cells but also adjacent pericytes.^{10,11,41–43} The influence of NO on migration of nonendothelial vascular cell types is varied with reports demonstrating promigratory effects of NO in smooth muscle⁴⁴ and anti-migratory effects in HSCs.²⁸ Furthermore, few studies have extended the influence of NO on migration to understand the actions of NO signals on specific cell biological correlates of migration such as actin membrane dynamics and small GTPase function. The present studies demonstrate that, contrary to its promigratory effects on endothelial cells and in some studies smooth muscle cells, NO inhibits Rac signals that drive formation of filopodia and ensuing LX2 migration.

Recent studies have highlighted the essential function of the PDGF system in the process of pericyte recruitment to tumor vessels with inhibition of this pathway importantly limiting tumor angiogenesis and growth.⁴³ Therefore, the inhibitory actions of NO on PDGF-dependent pericyte migration may have significant inhibitory effects on tumor vessel morphology, particularly in vascular beds such as liver, which are highly dependent on pericytes for angiogenesis, vascular extension, and remodeling. In this regard, the present studies provide a number of mechanistic advances in the membrane biology and signaling pathways that regulate motility and migration thereby advancing potential avenues for therapeutic interventions aimed at targeting HSCs for tumor anti-angiogenesis and other disease processes.

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