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p190RhoGAP links the β1 integrin/caveolin-1 mechano-signaling complex to RhoA and actin remodeling

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Abstract

Objective—Hemodynamic shear stress influences endothelial cell phenotype. Integrins and RhoA are essential components in the process that allow endothelial cells to adapt to flow. However, the signaling mechanisms that relay from integrins to RhoA are not well defined. We hypothesized that the β1 integrin/caveolin-1 signaling complex plays a role in shear stress regulation of RhoA activity through temporal regulation of p190RhoGAP.

Methods and Results—BAEC were subjected to laminar shear stress (10dynes/cm2) for up to 6 hrs. β1 integrin blockade inhibited Src-family kinases (SFK) and p190RhoGAP tyrosine phosophorylation observed following acute onset of shear stress. Depletion of caveolin-1 blocked the decline in p190RhoGAP tyrosine phosporylation observed at later time points through sustaining SFK activity. Manipulation of β1 integrin and caveolin-1 also altered shear-regulation of RhoA activity. More importantly, cells depleted of p190RhoGAP showed faulty temporal regulation of RhoA activity. Each of these treatments attenuated actin reorganization induced by flow. Similarly, stress fibers failed to form in endothelial cells exposed to enhanced blood flow in caveolin-1 knockout mice.

Conclusions—Our studies demonstrate that p190RhoGAP links integrins, caveolin-1/caveolae to RhoA in a mechanotransduction cascade that participates in endothelial adaptation to flow.

Keywords

caveolae; mechanotransduction; shear stress; p190RhoGAP; integrins

The hemodynamic environment in which an endothelial cell resides strongly influences cell morphology through regulation of cytoskeletal structures 1. Several, now classic studies 2–5, illustrate that in vitro and in vivo, actin stress fibers orient parallel to the direction of flow and are prominent in endothelial cells subjected to high shear velocities. These actin bundles reflect an adaptive response to shear stress which may aid endothelial cells in withstanding elevated hemodynamic stress, as in the case of hypertension.

While the influence of flow on regulating endothelial cell phenotype is now widely recognized, the fundamental process by which these cells detect and transduce fluid mechanical forces into biochemical signals is not completely clear. Past studies have

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described individual cellular elements with potential mechano-signaling properties including, ion channels 6, integrins 7, the glycocalyx 8, cilia 9, PECAM-1 10, various receptors for humoral compounds 11, the cytoskeleton 12 and the plasma membrane including lipid raft and caveolar subdomains 13,14. Interestingly, many of these link to similar sets of second messenger signaling molecules and regulate the same flow responses such as eNOS regulation of nitric oxide production and events downstream of ERK1/2 activation. These observations suggest that primary mechano-signaling elements likely engage in a substantial degree of interaction and cross-talk. Indeed, recent findings show that integrin/VEGFR2 15 and PECAM-1/VE-cadherin 16 associations form important mechano-signaling complexes.

From our own work, we found that a signaling network comprised of β1 integrin and caveolin-1 develops in response to shear stress. Acute shear stress applied to cultured endothelial cells resulted in integrin-dependent phosphorylation of caveolin-1 (pY14) via a Src-family kinase (SFK) 17. The phosphorylation of caveolin-1 served to recruit C-terminal Src-like kinase (Csk) to the integrin/caveolin-1 complex resulting in additional regulation of SFK activity and induction of myosin light chain (MLC) phosphorylation. Key components of this pathway were found to be recruited to caveolar microdomains and dependent upon the presence of the caveolin-1 protein 18.

The small GTPase, RhoA, is a key second messenger in the mechanotransduction pathway that allows endothelial cells to adapt to changes in hemodynamic shear forces. Shear stress applied to cultured endothelial cells temporally regulate RhoA activity, a process which depends upon upstream integrin activation 19,20. Moreover, the overexpression of either dominant negative or constituatively active RhoA prohibits induction of actin stress fibers and morphological restructuring 19 demonstrating that shear modulation of acute signaling events, such as temporal regulation of RhoA activity, is crucially linked to long term outcomes of exposing endothelial cells to flow. While a general relationship between RhoA and caveolin-1 enriched membranes have been described 21, whether caveolae microdomains, either alone or in concert with integrins, influence the mechanotransducing properties of RhoA requires experimental evaluation.

In addition to integrins and caveolin-1/caveolae as proximal regulators of RhoA, guanine nucleotide exchange factors (GEF's) and GTPase-activating proteins (GAP's) directly modulate RhoA activity through stimulating GDP/GTP cycling (i.e. on/off), respectively. Early stages of cell adhesion induce integrin ligation to the extracellular matrix (ECM) followed by a rapid decrease in basal RhoA activity. Several studies show that the suppression of RhoA activity following integrin engagements involves SFK-dependent phosphorylation and activation of a major GAP, p190RhoGAP 22. Interestingly, recent reports suggest that p190RhoGAP may regulate RhoA activity through its association with plasma membrane lipid raft domains 23,24. Based on these observations, we hypothesize that hemodynamic shear stress is translated via an integrin/caveolin-1 signaling complex which serves to temporally regulate a SFK/p190RhoGAP/RhoA axis and subsequent actin remodeling.

Methods

Antibodies and reagents

All general buffers and reagents were purchase from either Fisher Scientific or Sigma unless noted otherwise. SFK inhibitor PP2 and its negative control, PP3, were purchased from Calbiochem. The following primary antibodies were obtained from commercial sources: caveolin-1 and p190RhoGAP pAbs (BD-Transduction Labs); SFK and RhoA pAb's (Santa Cruz); β1 integrin mAb JB1A (Chemicon); myosin light chain and β-actin mAb's (Sigma);

myosin light chain di-phosphorylated pAb (Strategic Biosolutions); pY416 SFK pAb (Biosource); Anti-phosphotyrosine 4G10 (UpState); Horse-radish peroxidase conjugated anti-rabbit and anti-mouse secondary antibodies (Amersham).

Enhanced flow In Vivo

All procedures were performed according to an Institutional Animal Care and Use Committee (IACUC) protocol approved by Temple University. Briefly, wt and Cav1−/− mice (8 weeks old, Jackson Laboratories, Bar Harbor, ME) mice were anesthetized with a mixture of ketamine and xylazine and body heat maintained at 37°C with a heating pad. The right common carotid artery was exposed and ligated by closure of a suture looped around the vessel. Due to blood shunting, this procedure increases blood flow in the contralateral, left, common carotid artery and enhances formation of stress fibers within endothelial cells lining the vessel wall [3,25,26]. To verify flow changes, blood flow was measured at vessel midpoint using a VisualSonic Vevo 770 Doppler Ultrasound with a Scan Head (RMV 716). One day post-ligation, blood flow was enhanced by 50% in the left common carotid compared to sham operated animals. Blood flow through the common carotid arteries of caveolin-1 KO mice were similar to wild type matched animals and ligation induced the same increase in flow through the contralateral vessel. At the conclusion of the experimental time point, the mice were euthanized with sodium pentobarbital and vessels were harvested, fixed and immunolabeled.

Cell culture

Bovine aortic endothelial cells (BAEC), purchased from Cell Applications (San Diego, CA.), were grown in MCDB-131 culture medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals) and 0.04 mg/ml gentamicin sulfate and maintained at 37°C, 95% humidity, and 5% CO_2. All experiments were performed using cells below passage 8.

Caveolin-1, p190RhoGAP and Csk siRNA

Similar to our past reports [17,27], BAEC were transfected with caveolin-1, p190RhoGAP or Csk SMART pool siRNA or siScrambled control using DharmaFECT-1 (Dharmacon, Inc., Lafayette, CO) according to the manufacturer's protocol. Similar to our previous results, cells were used 48 hours post-transfection when expression levels of caveolin-1, p190RhoGAP and Csk were reduced by greater than 90% (Supplemental Fig. I).

Caveolin-1 pY14 peptide

As described in detail in our past work [17], caveolin-1 peptides spanning the pY14 region were constructed with the Y14 site either phosphorylated (pY14) or unmodified (Y14). Peptides consisting of amino acids 1–27 (MSGGKYVDSEGHLY*TVPIREQCNIYKPNNC, Genscript Corp.) of caveolin-1 with the tyrosine-14(*) either phosphorylated or unmodified were individually linked to biotinylated Penetratin (Qbiogene). Penetratin (300nM) coupled to either control or phospho-peptides were incubated with endothelial cells for 1 hour prior to shear stress. To determine the efficiency of peptide uptake, cells were incubated with streptavidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA) and biotinylated Penetratin was detected via the oxidation of diaminobenzidine (Supplemental Figure II).

In Vitro shear stress system

A parallel plate flow chamber (Streamer model, Flexcell Corp.) was used to subject endothelial cell monolayers to laminar shear stress at 10 dynes/cm^2, as detailed in our past work [17]. The parallel plate flow chamber (Streamer model, Flexcell Corp.) was connected to a recirculating flow circuit composed of a variable speed peristaltic pump, a fluid

capacitor that dampens pulsation, and a reservoir with culture medium. A computer operated Masterflex L/S pump was controlled with Streamsoft v1.0 software and produced fluid flow. Shear stress was determined by the equation, **shear stress = $6\mu Q/bh^2$**, where Q is the flow rate in cm^3/min, b is the channel width in cm, h is the channel height in cm, and μ is the dynamic viscosity of the fluid in dyne-s/cm^2. Prior to placement in the parallel plate apparatus, endothelial monolayers are acclimated for 4 hrs in "flow-media" consisting of MCDB-131 containing 1% FBS. Temperature was maintained at 37°C, and pH and oxygen levels were maintained in a 95% air /5% CO_2 humidified incubation chamber.

Immunoprecipitation

Briefly, p190RhoGAP monoclonal antibody was conjugated to sheep-anti mouse-coated paramagnetic dynabeads (Dynal). Endothelial cell lysates (250μg) were incubated with the antibody/bead conjugates for 4 hrs at 4°C. The bound fraction was separated from the unbound material, and processed for Western blot analysis.

RhoA activation assay

Following shear stress, cell lysates were added to Rhotekin-RBD GST beads (Cytoskeleton) for 1 hr at 4°C. GTP-RhoA associated with Rhotekin was determined by Western blot.

Western blotting

Endothelial cells were processed for Western blot analysis as described in our past work 17. Autorads were scanned, digitized and band intensities quantified using Image J software.

Caveolae immunoaffinity isolation

Caveolar vesicles were purified as described in our previous work 18. Briefly, static and shear exposed BAEC's were scraped into detergent-free Tricene buffer (250mM sucrose, 1mM EDTA, 20mM Tricene, pH 7.4) and processed to enrich plasma membranes. Plasma membranes were subsequently sonicated and incubated with anti-caveolin-1 conjugated goat anti-mouse IgG-coated magnetic beads (Dynal Biotech) for 1 hour at 4°C. Bound material, representative of caveolae vesicles, was separated magnetically from unbound, non-caveolar membranes.

Microscopy and quantification of stress fibers

Cell monolayers and carotid vessels were fixed (4% formaldehyde) and permeabilized with 100μg/mL saponin in HEPES buffer (10mM HEPES, 100mM KCl, 5mM $MgCl_2$), labeled with Phalloidin- AF488 (Molecular Probes) to visualize F-actin (stress fibers) and DAPI (nucleus). Images were captured using a Nikon Eclipse TE300 confocal microscope. Fluorescence signal intensity was quantified using EZ C1 3.90 Free viewer. Briefly, the average fluorescence intensity along a line connecting the edges of the nucleus between two paired endothelial cells was recorded. For each experiment (n=3), four random fields were chosen and 15 paired cells per field were unbiasly selected for analysis.

Statistical analysis

For each study, data was gathered from at least three independent experiments and pooled according to group. Mean and standard deviation were calculated and differences between groups analyzed with an unpaired two-tailed Student's *t* test or ANOVA with a post-hoc Tukey test using STATGRAPHICS 4.0 software (Statistical Graphics Corp). Differences between control and experimental groups were deemed significant at $p < 0.05$.

Results

Induction of actin stress fibers in endothelial cells under enhanced flow in vivo requires caveolin-1

Our past in vitro studies indicate that caveolin-1 and β1 integrin associate under enhanced flow conditions to form a signaling complex which regulates endothelial cytoskeletal elements. In order to evaluate key aspects of this mechanotransduction process in vivo, F-actin was labeled with a phallodin-fluorophore conjugate in endothelial cells residing in carotid arteries of wild type (wt) or caveolin-1 deficient mice (KO) following surgical enhancement of blood flow. In sham operated wt animals, F-actin was organized in dense peripheral bands and short stress fibers randomly oriented throughout most endothelial cells while F-actin in Cav1−/− mice appeared less developed (Figure 1). In response to flow, the F-actin signal increased 2-fold in wt vessels with the development of stress fibers which oriented parallel to the direction of blood flow. In carotid vessels of caveolin-1 knockout mice however, stress fibers failed to form and F-actin content of the endothelium was similar to sham operated animals.

Integrin/caveolae mechano-signaling complex regulates p190RhoGAP phosphorylation

To more effectively evaluate the molecular signaling mechanism that relay from the β1integrin/caveolin-1 complex to the actin cytoskeleton in response to flow, we exposed cultured endothelial cells to well defined fluid shear stress using a parallel plate apparatus. Our previous studies indicate that signaling components localized to an integrin- and caveolae-based mechano-signaling complex can spatially and temporally regulate shear-mediated SFK activity 17. A more complete evaluation of each element within this complex on shear-induced SFK activation revealed that β1 integrin and caveolin-1 serve to temporally regulate shear-induced SFK activity (Supplemental Figure III).

An important function of SFK is to propagate and amplify mechano-signals which influence cell structure in response to shear stress 28. To address the molecular events that bridge integrin/caveolin regulation of SFK's to endothelial cell adaptation to flow, we focused on signaling molecules that are influenced by SFK's and regulate downstream pathways that govern the cells cytoskeletal architecture, namely p190RhoGAP and RhoA. Figure 2 illustrates that following acute onset of shear stress, p190RhoGAP tyrosine phosphorylation initially increases followed by a decrease over time. Antibody blockade of β1 integrins prohibited these shear-induced phosphorylation events while both caveolin-1 siRNA and a caveolin-1 phospho-peptide prevented the decline in p190RhoGAP phosphorylation (Figs 2a, c and d, respectively). The data regarding both the caveolin-1 and caveolin-1 peptide experiments are consistent with their effects on enhancing SFK activity and thereby maintaining phosphorylation of p190RhoGAP. Lastly, inhibition of SFK with PP2, significantly decreased basal levels of p190RhoGAP phosphorylation and subsequent shear-induced tyrosine phosphorylation of p190RhoGAP. Taken together, these findings are the first to describe p190RhoGAP as a shear-sensitive signaling molecule.

Integrin/caveolae mechano-signaling complex temporally regulates RhoA activity

In a similar manner to the experiments described above, we evaluated shear-induced activity status of the p190RhoGAP target, RhoA. Figure 3 illustrates that RhoA activity initially declines from baseline levels followed by an increase at 30min exposure to shear stress. To determine whether integrins relay shear-induced signals to enhanced RhoA activity, cells were pretreated with JB1A. We found that JB1A significantly attenuated the activation of this small-GTPase in response to shear stress (Fig. 3a). We also found that depletion of caveolin-1 enhanced basal RhoA which was sustained at all shear stress time points (Fig. 3b). In cells expressing the cav-1 phospho-peptide, where both SFK and p190RhoGAP

phosphorylation is sustained, a marked inhibition of shear-induced RhoA activity was observed (Fig. 3c). Similar to caveolin-1 siRNA treatments, depletion of p190RhoGAP substantially elevated basal RhoA activity in endothelial cell monolayers (Fig. 3d).

Integrin/caveolae mechano-signaling complex regulates MLC phosphorylation and actin stress fiber formation

The phosphorylation of myosin light chain (MLC) is a critical event in the formation of distinct stress fibers which accompanies endothelial cell acclimation to shear stress. Our recent reports demonstrate that both integrin activation and caveolae/caveolin-1 are upstream of shear-induced MLC phosphorylation17. Similar to our past findings and consistent with the effects of JB1A on SFK, p190RhoGAP and RhoA, blockade of β1 integrin attenuated MLC phosphorylation (Fig. 4a). Similar results were observed with both the 3S3 and 6S6 β1 integrin blocking antibodies (kind gift from Dr. John Wilkins, Univ. of Manitoba) which target to alternative epitopes of the integrin molecule (data not shown). While loss of caveolin-1 enhanced SFK and RhoA activity, these events did not translate to phosphorylation of MLC (Fig. 4b). We also found that temporal regulation of SFK was important for proper phosphorylation of MLC since caveolin-1 phospho-peptide and Csk siRNA also attenuated shear-induced MLC phosphorylation (Figs. 4c and 4d). Last, depletion of p190RhoGAP, which activates RhoA, significantly enhanced baseline phosphorylation of MLC which increased modestly in response to shear stress (Fig. 4e).

Our in vivo findings demonstrated that caveolae/caveolin-1 are necessary cellular components for actin stress fiber formation in response to enhanced flow (Fig. 1). To evaluate the other components of the integrin/caveolae mechano-signaling pathway on actin reorganization, cultured endothelial cells were also examined for actin stress fiber formation following shear stress. Exposing endothelial monolayers to 6 hrs of laminar shear stress significantly enhanced F-actin labeling (Fig. 5) and formation of stress fibers (Supplemental Fig. IV). These events correlated with continued and progressive phosphorylation of MLC throughout the 6 hr shearing period (Supplemental Fig. V). Interestingly, RhoA activity was also sustained through a three hour observation time point but decreased by 6hrs (Supplemental Fig. V), perhaps indicating that the endothelial monolayers are adaptating to the flow environment. As in the case of acute responses to shear stress, JB1A blocked long term RhoA activity, MLC phosphorylation (Supplemental Fig. V) as well as stress fiber formation (Fig. 5 and Supplemental Fig. IV). Depletion of caveolin-1 also inhibited shear-induced MLC phosphorylation, enhanced F-actin labeling and the development of stress fibers in endothelial cell monolayers (Fig. 5 and Supplemental Fig. IV). Similar to the earlier observation time points, decreasing caveolin-1 protein expression enhanced the basal activity of RhoA through 6hrs of surveillance (Supplemental Fig. V).

Caveolar localization of p190RhoGAP and RhoA

We recently reported that shear stress induces the transposition of β1 integrin into caveolae plasma membrane domains where they participate in the formation of the integrin/SFK/phospo-caveolin-1/Csk signaling complex 18. To determine if the downstream elements examined here, namely p190RhoGAP and RhoA are present in caveolar vesicles, we immunoaffinity isolated caveolae from sheared and non-sheared endothelial cells. Figure 6 shows that under static culture conditions, very little p190RhoGAP is present in caveolae. Consistent with other reports, 21،29 and our past studies, 27 a pool of RhoA is constitutively targeted to caveolae domains. Following the onset of shear stress, p190RhoGAP was detected in caveolae with significant transposition observed by 5 min post-shear while expression level of RhoA remained unchanged. The association of p190RhoGAP appeared to be transient since we observed a significantly loss of p190RhoGAP in the caveolae

fraction after cells had been subjected to 30mins of shear stress. On the contrary, the RhoA content increased over 5-fold in caveolae domains at this later time point.

Discussion

Subjecting endothelial cells to acute increases in shear stress, both in vivo and in vitro, sets off a signaling cascade which results in the alteration of cell morphology that serves the endothelium in adapting to new flow environments. A major feature of this process is the reorganization of the cytoskeletal network and the development of actin stress fibers. While the precise signaling mechanism which regulates this process is not fully understood, the temporal regulation of RhoA activity appears to be essential for proper cytoskeletal realignment and adjustment of endothelial cells to changes in shear 19,30,31. Several studies have also demonstrated that shear-induced RhoA activation is secondary to integrin activation however, the mechano-signaling mechanisms that link integrins to RhoA and actin remodeling remains unclear. Past work from our laboratory indicate that the shear-induced phosphorylation of myosin light chain, an event that is associated with actin stress fiber formation, is mediated by a β1 integrin/caveolin/Csk signaling complex and depend on the presence of caveolar organelles 17,18. In this study, we systematically evaluated whether this mechano-signaling complex constitutes a bridge from integrins to RhoA and stress fiber formation in response to shear stress.

Consistent with other reports19, we observed an initial reduction in basal RhoA activity followed by an increase in activity through 3hrs exposure to shear stress before returning towards baseline. We found that β1 integrin participated in this temporal pattern of shear-induced RhoA activity since significant changes in activity were not observed in cells that were pretreated with a β1 integrin blocking antibody, JB1A (Fig. 3a and Supplemental Fig. V). These findings are in agreement with events described for regulation of RhoA activity during the process of cell adhesion where β1 integrin ligation to ECM components activates signaling pathways which first suppress RhoA activity and then, during later stages of cell spreading, is enhanced 22,32. Thus, cell adhesion and mechanotransduction processes appear to share similar signaling properties.

Next, we examined the influence of SFK's on RhoA activity given that the formation of a β1 integrin/caveolin-1/Csk signaling complex was crucial for the temporal regulation of Src-family kinase activity and subsequent relay to important RhoA targets such as myosin light chain (MLC). As we previously described 17, SFK activation in response to shear stress was rapid, yet transient (Supplemental Fig. III). In experiments where SFK activity was sustained either through depletion of caveolin-1 or sequestration of Csk with a phosphorylated N-terminal caveolin-1 peptide, the temporal regulation of RhoA activity by shear stress was significantly altered (Fig. 3). These findings illustrate that SFK's play a role in regulation of RhoA activity by shear stress. Since SFK have been shown to influence RhoA activity through SFK-dependent phosphorylation of signaling intermediates in response to integrin engagement 22, we evaluated the pattern of p190RhoGAP phosphorylation in response to shear stress. Following the onset of shear stress, p190RhoGAP phosphorylation increased followed by de-phosphorylation events that correlated with GDP/GTP-loading of RhoA, respectively. More importantly, the depletion of p190RhoGAP protein expression resulted in the loss of temporal regulation of RhoA activity induced by shear stress (Fig. 3d). Together, these findings suggest that p190RhoGAP function is sensitive to shear stress and that RhoA is a direct p190RhoGAP target in mechanotransduction.

It has been established that changes in the temporal sequence of RhoA activity observed in endothelial cells during the first hour of exposure to shear stress is critical for long term

adaptive responses such as actin reorganization and cell alignment 19. Here, we show that, in addition to serving as proximal signaling elements that govern RhoA activity, β1 integrin, caveolin-1 and p190RhoGAP are necessary for the formation of stress fibers induced by prolonged exposure to shear stress (Figs. 1 and 5 and Supplemental Fig. IV). Collectively, the studies demonstrate that maintaining the shear-induced off/on activity status of RhoA is essential for endothelial cells to morphologically adapt to flow. Moreover, our findings advance this concept by describing a molecular signaling mechanism that relay from integrins to RhoA to the cytoskeleton with p190RhoGAP being implicated in the shear stress responses in endothelial cells for the first time.

It is becoming increasingly clear that signaling transduction is often compartmentalized within particular cellular microdomains. We previously reported that plasma membrane caveolae served as sites for the recruitment and formation of a β1 integrin signaling complex that communicated with downstream elements involved in endothelial morphological adaptation to flow 17. In the current study, we also discovered that both p190RhoGAP and RhoA associated with caveolae, although to varying degrees following acute exposure of endothelial cells to shear stress. Similar to past reports 23›24, we found that a pool of RhoA was basally targeted to caveolae and RhoA agonists, including shear (this study), induces further recruitment of this GTPase to these domains. In contrast, we found very little p190RhoGAP present in caveolae derived from control, non-sheared, endothelial cells. However, shear stress induced recruitment of p190RhoGAP to caveolae at an early time point when RhoA activity is depressed. When RhoA became most active, p190RhoGAP appeared to be released from these domains. While we did not measure RhoA activity status in caveolae, per se, the observed compartmentation of both p190RhoGAP and RhoA suggest that caveolae serve as platforms that allow for temporal regulation of RhoA activity in response to shear stress. This concept is supported by recent evidence showing p190RhoGAP/RhoA interactions relative to the more generalized lipid raft domains of the plasma membrane 23. In those studies, RhoA is most active in non-adherent cells and p190RhoGAP was observed to reside mainly outside of lipid raft where its activity was downregulated. Upon adhesion, p190RhoGAP accumulated in raft domains where it became active and able to suppress RhoA activity. The similarity in observations between these studies and ours suggest again that initiation of shear stress activates signaling events that may recapitulate those of cell adhesion.

Interestingly, depletion of caveolin-1 elevated the activity of RhoA and SFK's over baseline (Supplemental Fig. III) in our system. While these observations are consistent with the function of caveolin-1 as a signaling molecule inhibitor, the rise in basal SFK and RhoA did not translate in larger changes in the cells phenotype such as remodeling of the actin cytoskeleton (Fig. 5 and Supplemental Fig. IV). On the surface this may seem surprising but if one considers that each of these signaling molecules can be compartmentalized within caveolae microdomains (Fig. 6), along with several of their downstream targets, then the loss of caveolin-1, and more importantly caveolae as a consequence, would be expected to disrupt efficient signal propagation from these sites. In support of this concept and consistent with previous observations29, we found that disruption of caveolae and lipid rafts with cholesterol depleting agents, such as methyl-β-cyclodextrin, shift SFK and RhoA from these membrane microdomains to other cellular compartments (Supplemental Fig. VI) and provide additional evidence for the localization of these mechanosensitive signaling molecules to rafts and caveolae.

While other integrin subtypes, GTPases and second messengers have been implicated in mechanotransduction pathways that contribute to endothelial adaptation to flow 33, our collective studies in this area indicate that shear stress also induces the formation of a caveolae-based signaling complex where mechanically sensitive integrins translocate to

caveolae and associates with caveolin-1. This complex appears to regulate endothelial functions that participate in vascular remodeling events initiated by altered flow. In support of this concept, recent studies using endothelial cell specific heterozygous β1 integrin gene depletion (which reduced β1 integrin protein expression in endothelial cells by 40%) show abnormal vascular remodeling in response to experimentally altered blood flow in vivo 34. Our experiments in endothelial cell cultures and animal models of flow enhancement demonstrate that flow-induced remodeling of the actin cytoskeleton requires caveolin-1/caveolae. Taken together, these studies demonstrate that both caveolin-1/caveolae and β1 integrin are linked in their functions as mechanotransduction elements within endothelial cells and may serve as appropriate targets to modulate endothelial sensitivity to hemodynamic forces.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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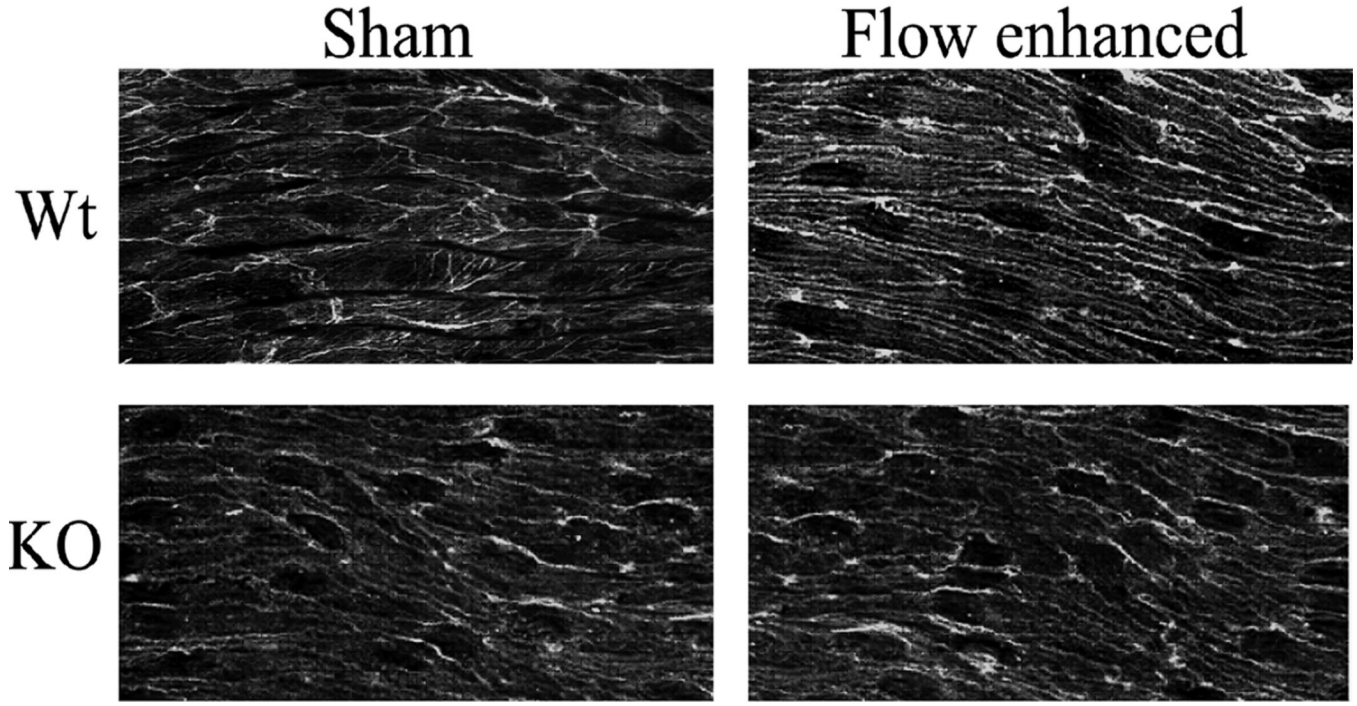
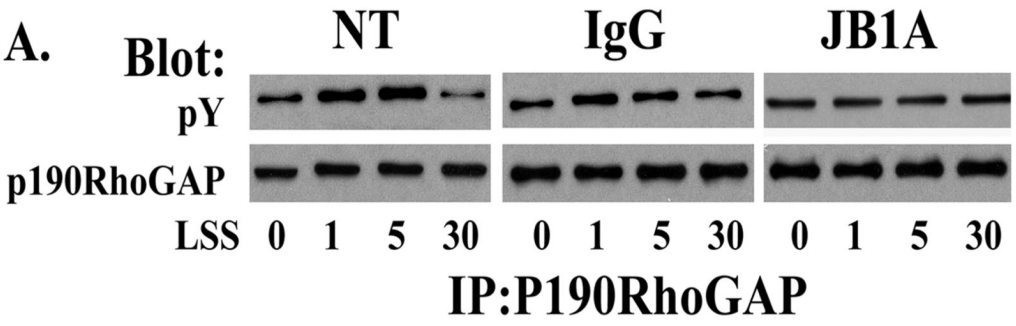
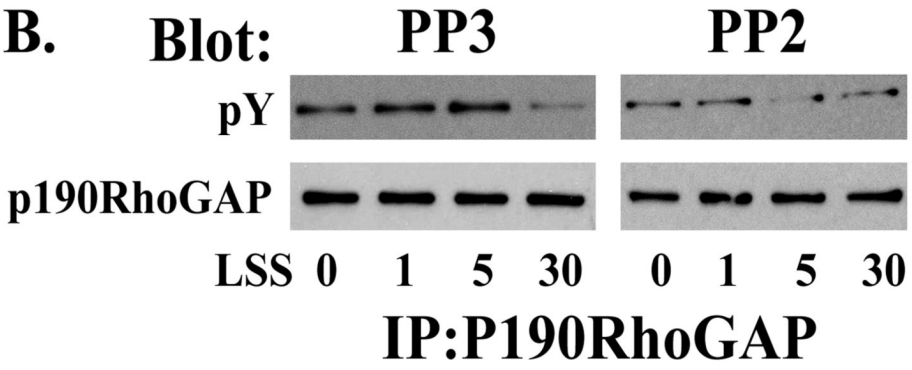
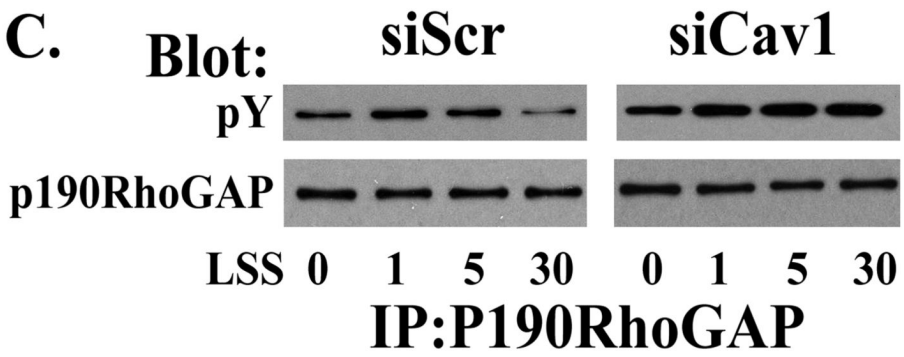


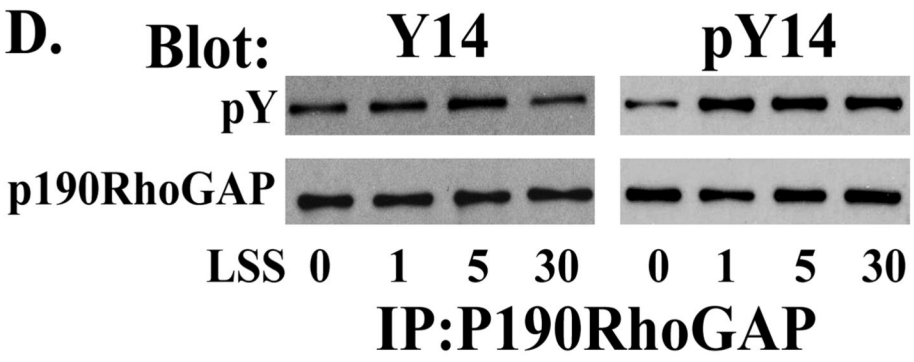
Figure 1. Induction of actin stress fibers in endothelial cells under enhanced flow in vivo is mitigated in caveolin-1 knockout mice

The right common carotid artery of wild type (Wt) or caveolin-1 knockout (KO) mice was either ligated to enhance flow through the left common carotid vessel or left open. One day after ligation, the mice were euthanized and the left common carotid vessels excised, fixed and the actin cytoskeleton (F-actin) labeled with Phallodin-AF488 (Molecular Probes). Each vessel was then splayed open and a coverslip placed over the luminal side of the vessel wall. Confocal microscopy was used to image the endothelial cell cytoskeleton. Images are representative of experimental results and from one of 3 separate studies.









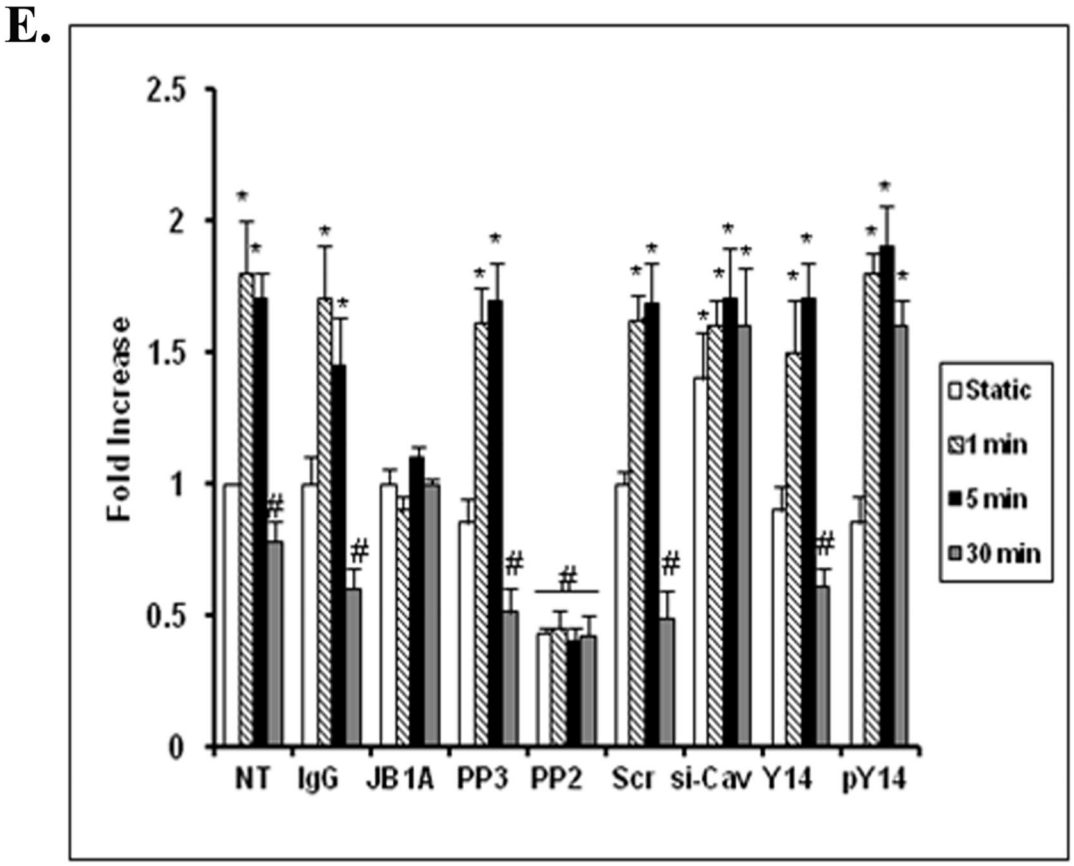
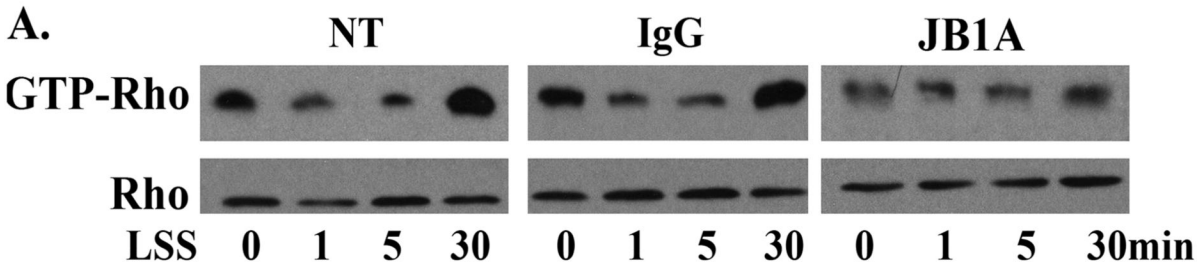
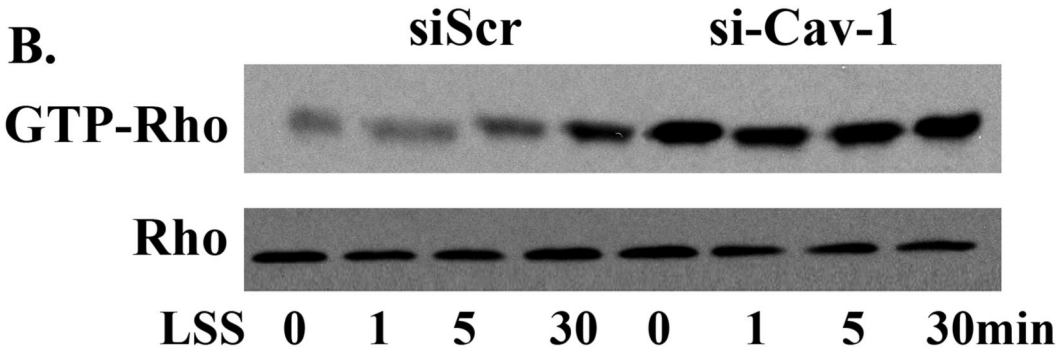


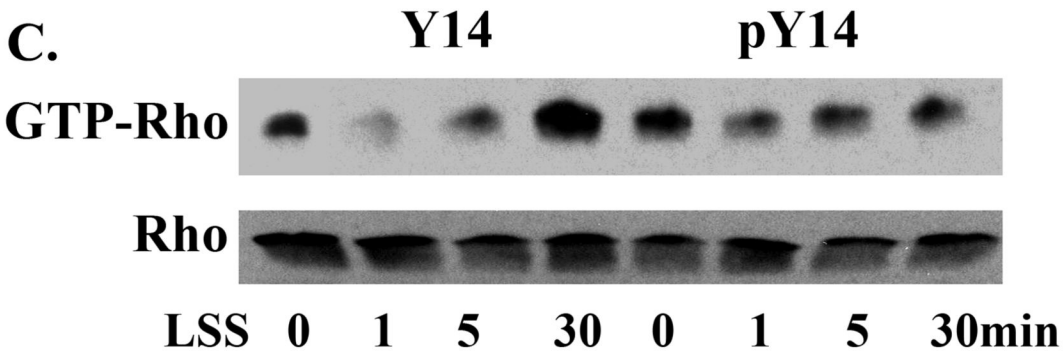
Figure 2. Shear stress-induced phosphorylation/de-phosphorylation of p190RhoGAP

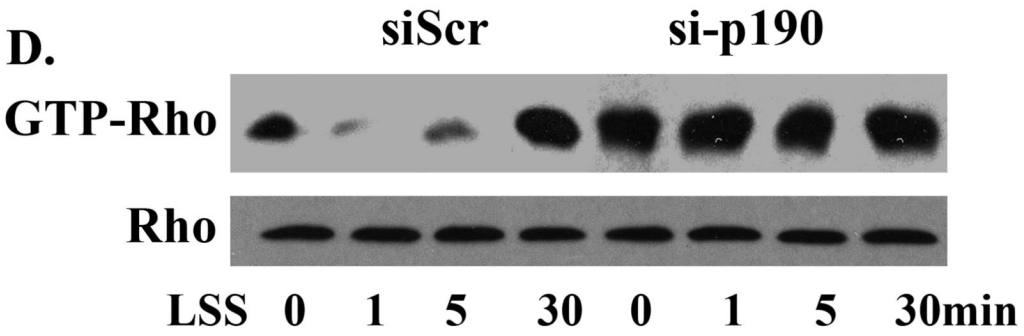
BAEC's were subjected to laminar shear stress (10dynes/cm2) for indicated time. Immunoprecipitated p190RhoGAP was probed for phospho-tyrosine (pY) content. To examine upstream mediators associated with the integrin/caveolae mechano-signaling complex, cells were pretreated with either IgG or JB1A antibodies (A), the SFK inhibitor PP2 or its negative control PP3 (B), caveolin-1 siRNA or its scrambled control (C) or phosphorylated (pY14) or non-phosphorylated (Y14) caveolin-1 peptides (D). Blots are representative of one of 3 independent experiments and (E) is the densitometric quantification of Western blots for each group at each shear time point where * indicates

significant enhancement (P value <0.05) over static, non-treated (NT) control samples and # represents significant (P value <0.05) reduction compared to the control group.









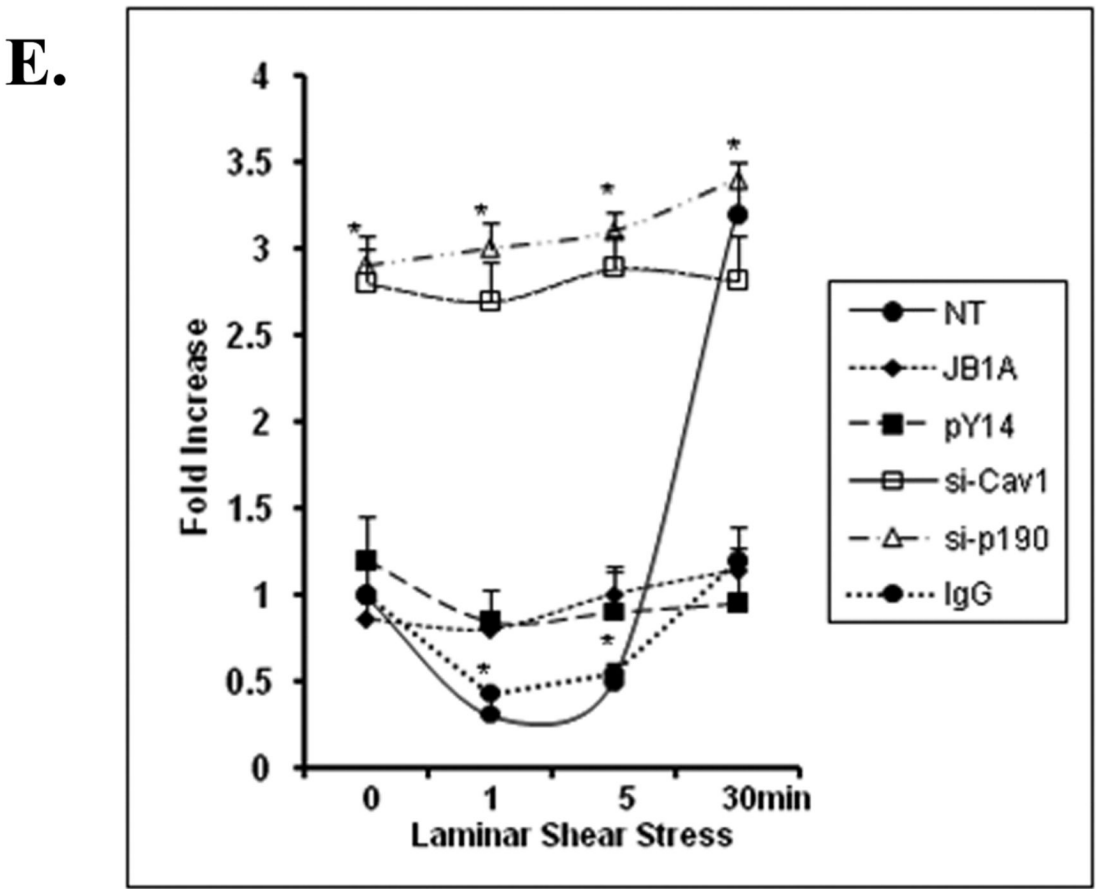
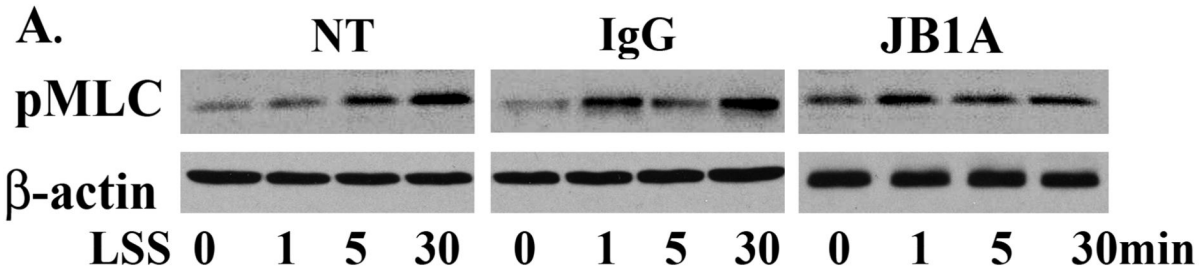
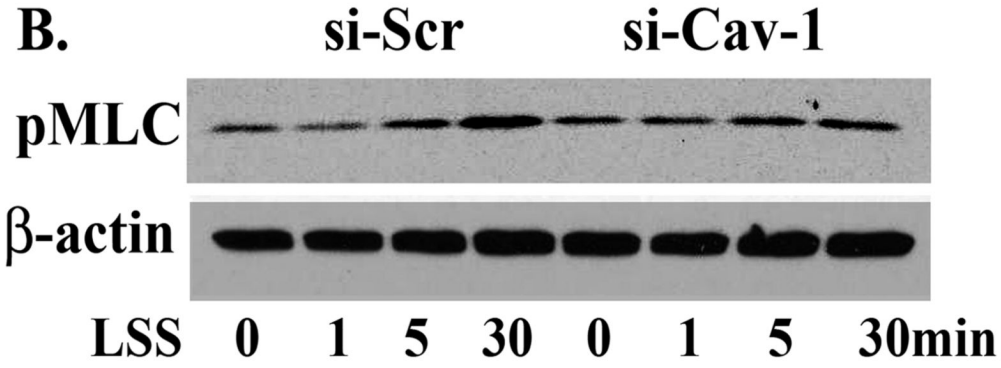
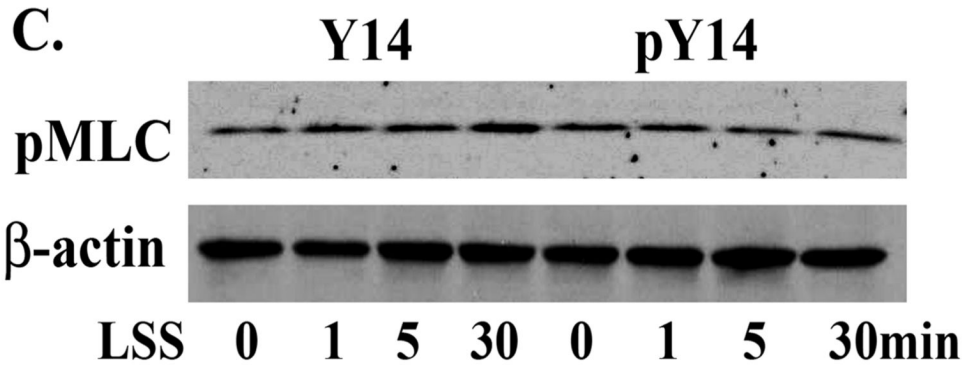


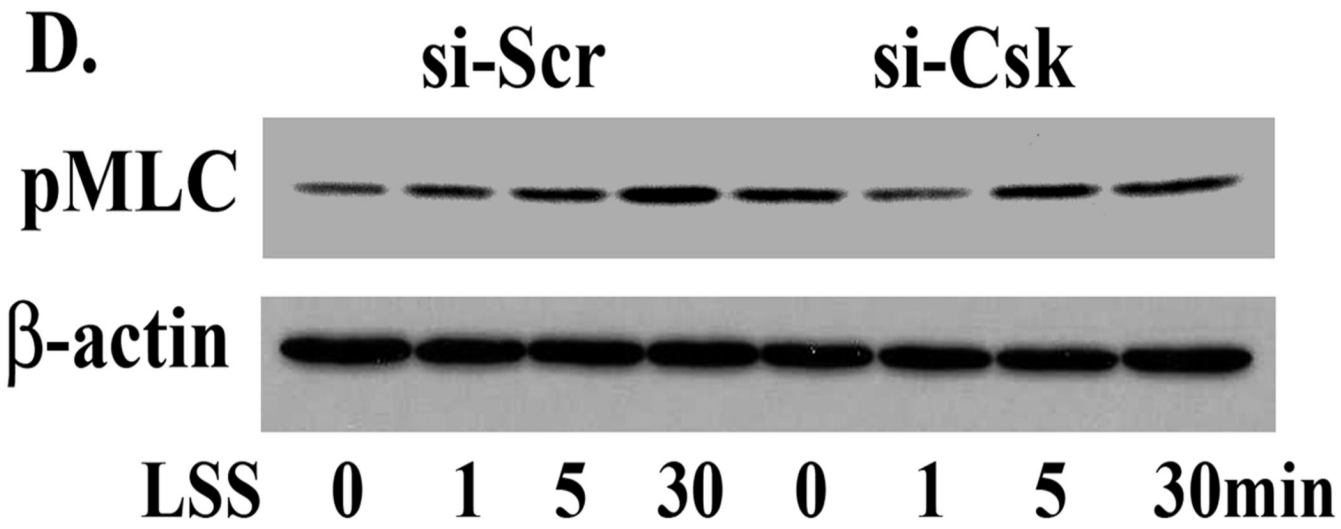
Figure 3. RhoA activity in response to shear stress

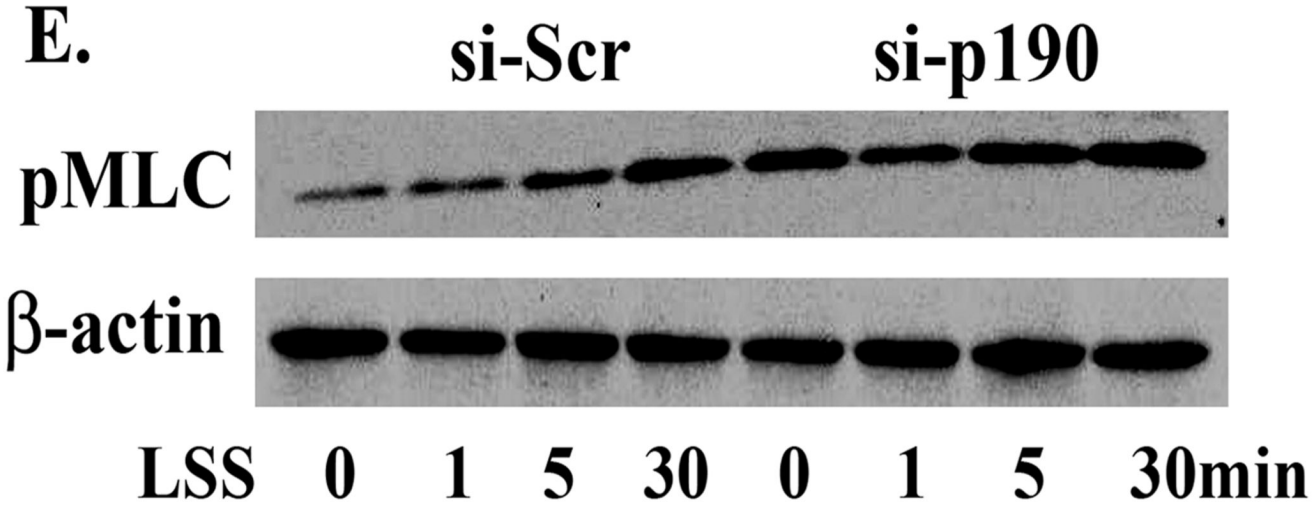
BAEC monolayers were untreated or pretreated with either IgG or JB1A (A), caveolin-1 siRNA (B), caveolin-1 peptides (C) or p190RhoGAP siRNA (D). Cells were exposed to 10 dynes/cm2 of shear stress for time indicated. RhoA activity levels were determined utilizing a rhotekin binding assay to isolate GTP bound RhoA from lysates. Immunoblots are representative of results acquired from 4 separate experiments. (E) Graphic representation of RhoA activity with and without (NT) the various pretreatments followed over 30 minutes of exposure to shear stress (*P value <0.05).











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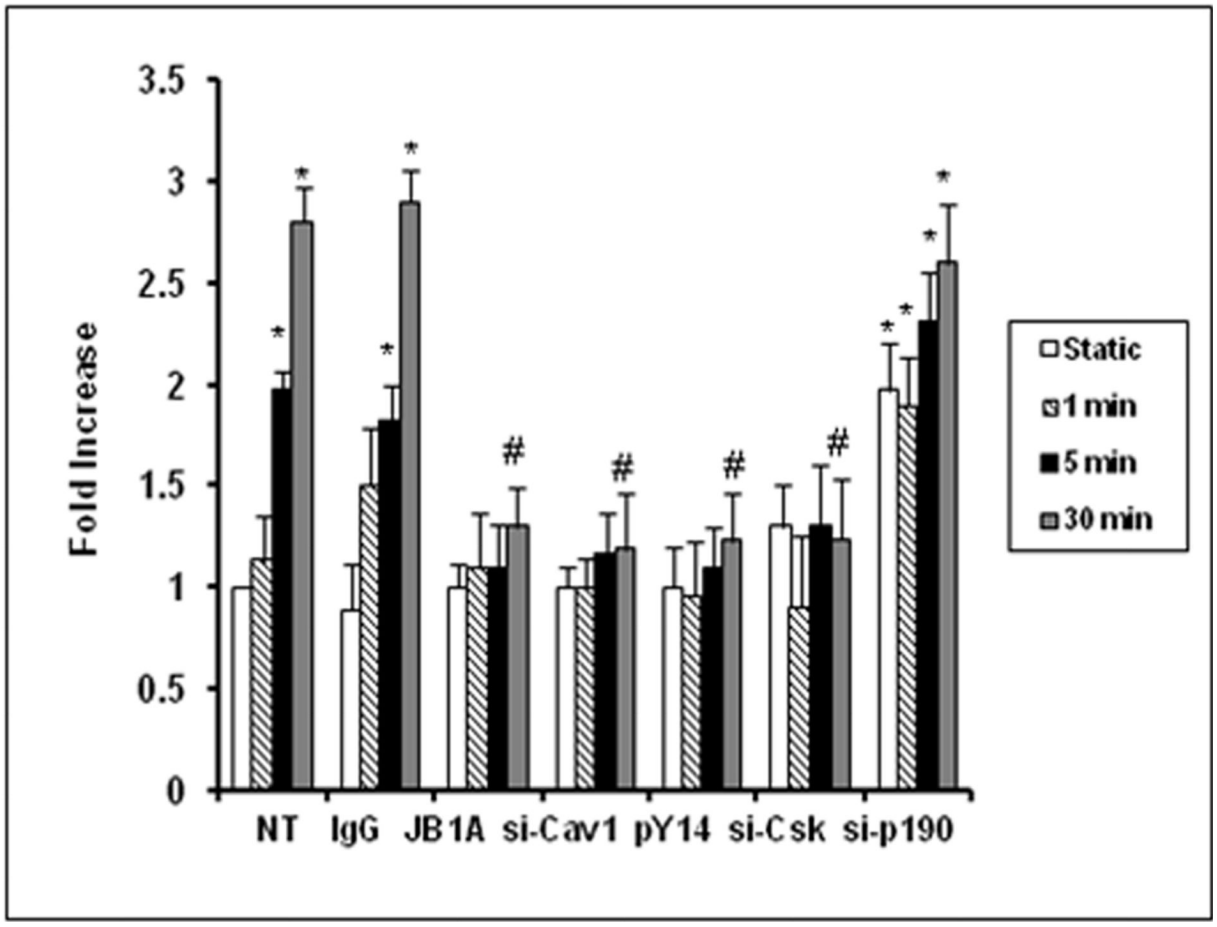

Figure 4. Shear Stress-enhanced Myosin Light Chain phosphorylation

Shear stress (10dynes/cm2) stimulated phosphorylation of MLC. To evaluate each component of the integrin/caveolae mechano-signaling complex, cells were pretreated with either JB1A (A), caveolin-1 siRNA (B), caveolin-1 peptides (C), Csk siRNA (D) or p190RhoGAP siRNA (E) and their appropriate controls. β-actin verified equal loading. The Western blots are illustrative of results observed in 3 independent experiments. (E) Histographic depiction of Western analysis where * indicates significant enhancement (P value <0.05) over static, non-treated (NT) control samples and # represents significant (P value <0.05) reduction compared to the 30 minute time point of the control group.

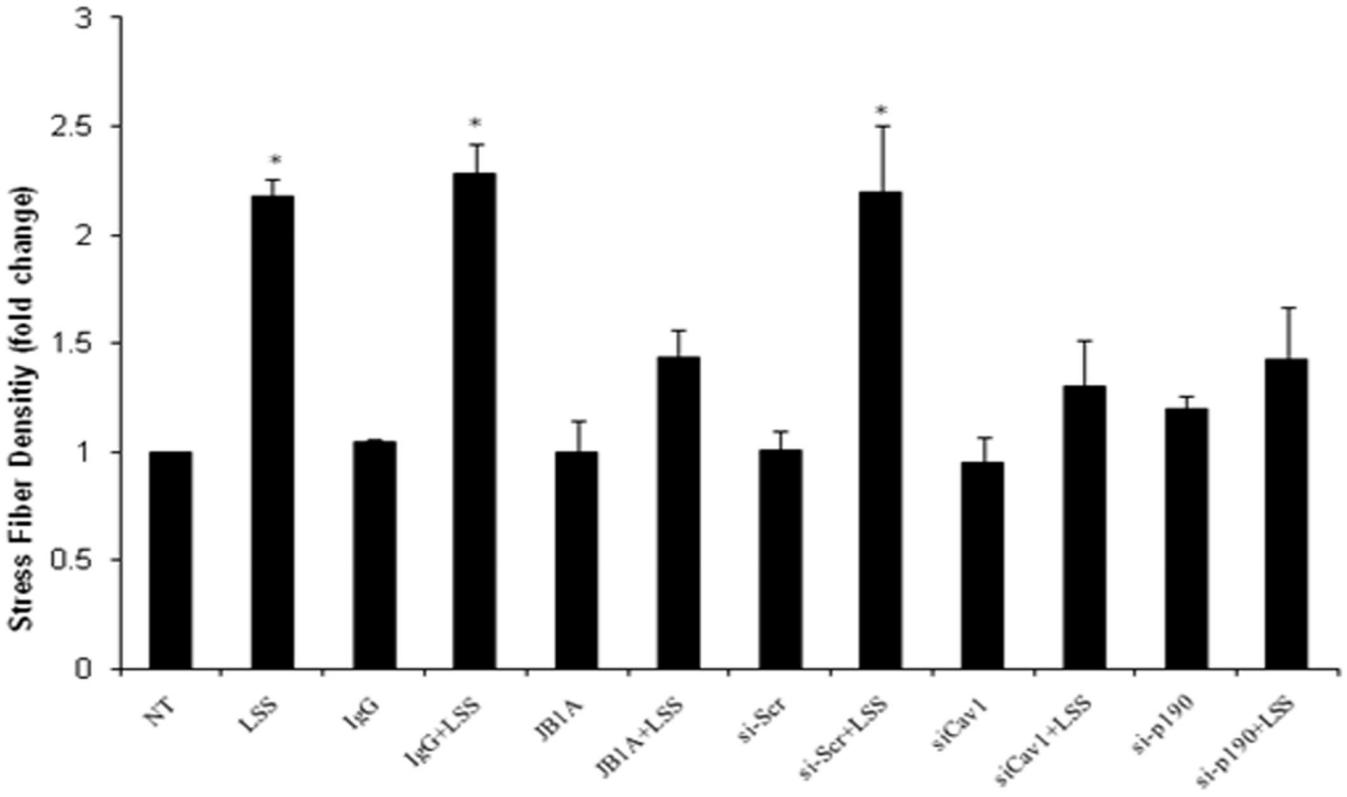

Figure 5. Shear stress induces actin stress fiber formation

BAEC were pretreated as indicated and either cultured under static, no-flow conditions or exposed to shear stress (10 dynes/cm2) for 6hrs. Cells were fixed and F-actin labeled with a phalloidin-fluorophore conjugate. Stress fibers were quantified as described in the METHODS and the data expressed in histogram form. (* signifies P value <0.05)

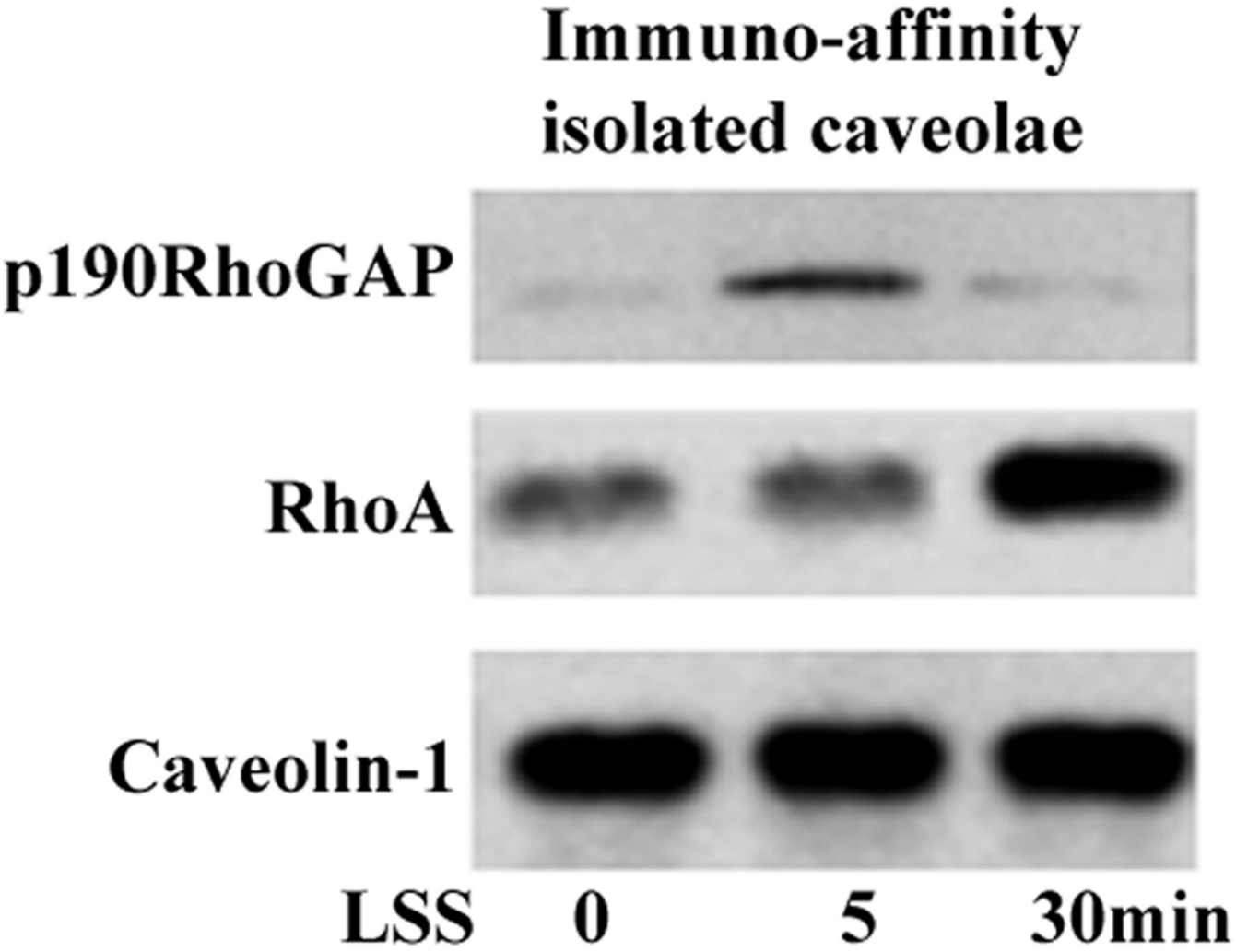

Figure 6. Association of p190RhoGAP and RhoA in endothelial cell caveolae in response to shear stress

Caveolae derived from endothelial cell cultures exposed to acute laminar shear stress (LSS) for up to 30 minutes were probed for 190RhoGAP and RhoA protein expression by Western blot analysis. Shear stress induced a rapid increase in p190RhoGAP content within caveolae (3.2 fold +/− 0.7 at 5 mins compared to the 0, no flow, time point) while RhoA expression levels remain unchanged. At 30 minutes of shear stress, p190RhoGAP levels returned to baseline while the RhoA signal became markedly enhanced (5.3 fold +/− 1.1) compared to both the 0 and 5 min shear exposure times. Shown is a representative immunoblot from one of three independent experiments.