



## Pulsatile equibiaxial stretch inhibits thrombin-induced RhoA and NF- $\kappa$ B activation

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### ABSTRACT

This study investigated interactions between the effects of mechanical stretch and thrombin on RhoA activation in rat aortic smooth muscle cells (RASMC). Equibiaxial, pulsatile stretch, or thrombin produced a significant increase in RhoA activation. Surprisingly, in combination, 30 min of stretch inhibited the ability of thrombin to activate RhoA. NO donors and 8-bromo-cGMP significantly inhibited thrombin-induced RhoA activation. Interestingly, the nitric oxide synthase (NOS) inhibitor L-NAME increased basal RhoA activity, suggesting that NOS activity exerts a tonic inhibition on RhoA. Stretching RASMC increases nitrite production, consistent with the idea that NO contributes to the inhibitory effects of stretch. Thrombin stimulates MAP kinase and NF- $\kappa$ B pathways through Rho and these responses were blocked by 8-bromo-cGMP or stretch and restored by L-NAME. These data suggest that stretch, acting through NO and cGMP, can prevent the ability of thrombin to stimulate Rho signaling pathways that contribute to pathophysiological proliferative and inflammatory responses.

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The role of Rho GTPases in regulation of cell morphology [1], vascular smooth muscle contraction [2], and cell migration [3] is well recognized. Equally important but less well studied is the involvement of RhoA in cell proliferation [3,4] and inflammatory responses [5,6]. Enhanced proliferative and migratory capacity of vascular smooth muscle cells (VSMC), coupled with an increased predisposition toward inflammation, characterize many blood vessel diseases, and growing evidence suggests that alterations in RhoA expression, activity, and/or signaling play a role in a subset of vascular disorders [7].

NOS mediated NO production and activation of the guanylyl cyclase-cGMP pathway have been widely demonstrated to regulate vascular smooth muscle contraction, proliferation, and migration [8]. In contrast to the stimulatory effects of RhoA, the effect of activating the NO pathway is to inhibit each of these responses. Increasing Rho activity has been shown to reduce eNOS mRNA stability [9], and it has been suggested that the effects of statins to upregulate both eNOS and iNOS result from Rho inhibition [9,10].

The G protein-coupled receptors (GPCRs) that signal through the G<sub>12/13</sub> family of heterotrimeric G proteins have well-documented effects on RhoA activation. Prominent among these are

the receptors for thrombin, lysophospholipids, and thromboxane A<sub>2</sub> [11]. Our previous reports demonstrated that acute stimulation of RASMC with thrombin produced significant increases in membrane-associated RhoA, as well as RhoA-[<sup>35</sup>S]GTP $\gamma$ S binding and RhoA activation [3,12]. In addition to agonist stimulation of Rho pathways, mechanical forces also regulate Rho [13–15]. Numaguchi and colleagues have shown that cyclic (i.e., pulsatile), mechanical stretch with a magnitude of 20% activates Rho in RASMC [14] and our laboratory demonstrated that static stretch-induced Rho activation in neonatal rat ventricular myocytes [15]. While the mechanisms for activation of RhoA in response to GPCR stimulation or mechanical forces have been investigated [13,16,17], the interaction of mechanical and hormonal stimuli in regulating RhoA activation has not been addressed.

VSMC *in vivo* are exposed to constant pulsatile stretch as a result of the cardiac cycle and are simultaneously regulated by circulating and locally derived neurohumoral factors. In this study, we examined the interactions between pulsatile, equibiaxial stretch, and thrombin, an agonist that elicits RhoA activation and Rho-dependent responses in RASMC [3,4]. The data provide evidence that stretch can inhibit RhoA activation by acting through NOS/cGMP, thereby attenuating agonist-induced Rho-dependent signaling pathways involved in cell proliferation and gene expression.

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## Methods

**Cell culture.** RASMC were isolated and cultured from the thoracic aorta of adult, male Sprague–Dawley rats as previously described [3]. The cells were maintained in high glucose DMEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 20% BSA in a 5% CO<sub>2</sub> incubator. Cells between passages three and eight were passed 4–7 days prior to assay, grown to 50–75% confluence, and serum-starved overnight.

**Pulsatile equibiaxial stretch.** RASMC were stretched using a device previously described [18,19]. Briefly, silicone membranes were attached to polycarbonate chambers with O-rings. The chambers were sterilized with UV light and coated with 1 µg/cm<sup>2</sup> fibronectin (Sigma, St. Louis, MO) overnight. RASMC were seeded onto the membranes at 3125 cells/cm<sup>2</sup>, grown until 50–60% confluent, and serum-starved overnight prior to stretching. All controls were time-matched, sham controls. The stretching device was set to produce a 10% linear stretch at a frequency of 1 Hz. Shear stresses due to the movement of fluid in the stretch chamber are estimated to be no more than 0.2 dynes/cm<sup>2</sup> and do not affect the signaling pathways investigated in this study [20]. The entire device was placed inside an incubator to maintain 100% humidity, 5% CO<sub>2</sub>, and 37 °C.

**Immunoblot Analysis.** RASMC were lysed with a buffer containing 10 mM Tris–HCl, 150 mM NaCl, 2.5 mM EDTA, 10% glycerol, 1% Igepal/NP-40, 50 mM NaF, 20 mM Na pyrophosphate, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 mM PMSF. Lysates were clarified by centrifuging and protein concentration was obtained by the Bradford protein assay. Following SDS–PAGE and protein transfer, the PVDF membranes were blocked with 5% milk or 3% BSA in TBS/Tween 20, and then exposed to either p-ERK antibody (Cell Signaling Technology, Beverly, MA) or p-IκBα antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. Following incubation with the appropriate secondary antibody, proteins were detected using enhanced chemiluminescence and analyzed by gel documentation imaging.

**RhoA activation.** RASMC were stretched for various times as indicated and/or exposed to vehicle or thrombin for 3 min prior to washing with PBS and lysis as previously described [12]. Lysates were subjected to affinity precipitation assay using the GST-fusion protein of the Rho binding domain of rhotekin (a generous gift from Dr. Martin Schwartz, The University of Virginia, Charlottesville, VA). The samples were then immunoblotted for active RhoA as previously described [12].

**Nitrite Assay.** One day before the experiment, RASMC were serum-starved overnight with phenol red-free DMEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin, 0.1% BSA, and 1.5 mM L-arginine. On the following day, cells were exposed to 10% pulsatile, equibiaxial stretch or thrombin for the times indicated. Nitrite in the media was measured using a Greiss reagent system (Promega) as previously described [21], and the absorbance of the samples was determined at 520 nm.

**Statistical analysis.** Data were analyzed by one-way ANOVA or Student's *t*-test where appropriate. For ANOVA, post-analysis was performed with the Tukey test using *p* < 0.05.

## Results

### *Pulsatile, equibiaxial stretch inhibits thrombin-induced RhoA activation*

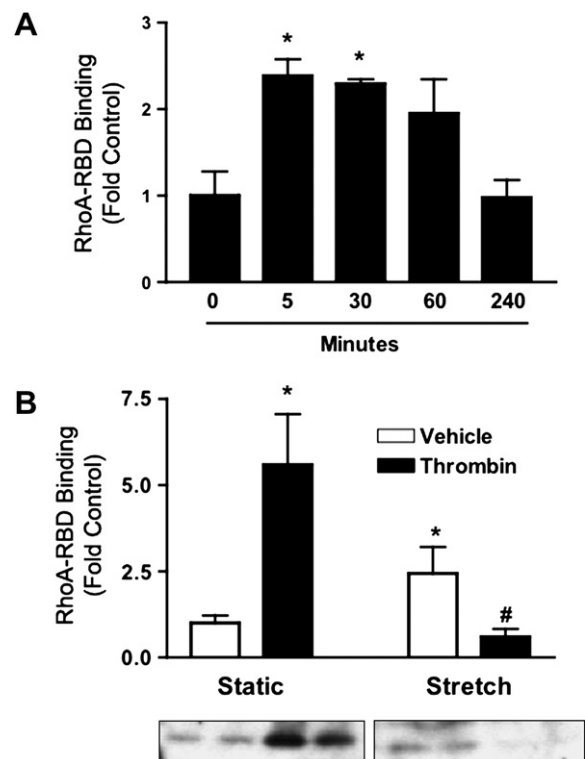
To examine the effect of mechanical stretch on RhoA activation, RASMC were harvested after 5 min to 4 h of stretch and subjected to rhotekin RBD affinity precipitation assay [12]. Five or 30 min of stretch produced modest but significant increases

in RhoA activation, which diminished by 1 h of stretch and completely returned to baseline levels by 4 h (Fig. 1A). Thrombin alone produced a robust fivefold increase in RhoA activation (Fig. 1B), consistent with previous observations [3,12]. Interestingly, when cells had been pre-exposed to stretch for 30 min, the addition of thrombin did not further activate RhoA, but instead stretch fully prevented thrombin-induced activation of RhoA (Fig. 1B).

### *The NO-cGMP pathway inhibits thrombin-induced RhoA activation*

Inhibition of thrombin-induced RhoA activation could also be elicited by stimulation of the NO-cGMP pathway. Pretreatment of RASMC with either a NO donor (PAPA-NONOate 250 µM) or 8-bromo-cGMP (500 µM) for 4 h led to a significant reduction in thrombin-induced RhoA activation (Fig. 2A). Conversely, inhibition of NOS by L-NAME treatment (10 mM) for 24 h led to a significant increase in basal levels of active RhoA (Fig. 2B).

Since mechanical forces such as shear stress have been reported to increase NO release from endothelial cells [22], we postulated that pulsatile, equibiaxial stretch might increase NO release from vascular smooth muscle cells. After 30 min of stretch, RASMC produced a significant increase in nitrite production (Fig. 2C) indicative of NOS-generated NO production. Thus, NO could attenuate the rise in RhoA activation in response to stretch and could contribute to the inhibition of thrombin-induced RhoA activation. Nitrite production under basal state and induced by stretch was L-NAME-sensitive (data not shown), indicating that NOS is responsible for the observed nitrite accumulation.



**Fig. 1.** Mechanical stretch stimulates RhoA activation, but inhibits thrombin-induced RhoA activation. (A) The increase in active RhoA following mechanical stretch of cells for 5–240 min. (B, left panel) The marked increase in RhoA activation by thrombin (12 nM for 3 min). (B, right panel) 30 min of pre-stretch reversed the effect of thrombin on RhoA activation from stimulation to inhibition. Data represent means and standard errors from three experiments. \**p* < 0.01 vs control. #*p* < 0.01 vs static thrombin.

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