Thromboxane Induced Actin Polymerization in Hypoxic Pulmonary Artery is Independent of Rho

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ABSTRACT

Rationale: Actin polymerization (APM), regulated by Rho GTPases, promotes myocyte force generation. Hypoxia is known to impede postnatal disassembly of the actin cytoskeleton in pulmonary arterial myocytes. We compared basal and agonist-induced APM in myocytes from pulmonary artery (PA) and descending aorta (Ao), under hypoxic and normoxic conditions. We also examined effects of thromboxane challenge on force generation and cytoskeletal assembly in resistance PA and renal arteries from neonatal swine with PPHN induced by 72 hour normobaric hypoxia, compared to age-matched controls.

Methods: Synthetic and contractile phenotype myocytes from neonatal porcine PA or Ao were grown in hypoxia (10% O₂) or normoxia (21% O₂) for 7 days, then challenged with 10⁻⁶M thromboxane mimetic U46619. F/G actin ratio was quantified by laser-scanning cytometry and by cytoskeletal fractionation. Thromboxane receptor (TP) G-protein coupling was measured by immunoprecipitation, probing for Gαq, G12 or G13; RhoA activation by Rhotekin-RBD affinity precipitation; LIMK and cofilin phosphorylation by Western blot. Isometric force to serial concentrations of U46619 was measured in muscular pulmonary and renal arteries from PPHN and control swine; APM was quantified in fixed contracted vessels.

Results and Conclusion: Contractile PA myocytes exhibit marked Rho-dependent APM in hypoxia, with increased active RhoA and LIMK phosphorylation. Their additional APM response to U46619 challenge is independent of RhoA, reflecting decreased TP association with G12/13 in favor of Gαq. In contrast, hypoxic contractile Ao myocytes polymerize actin modestly, and depolymerize to U46619. Both basal APM, and the APM response to U46619, are increased in PPHN PA. APM corresponds with increased force generation to U46619 challenge in PPHN PA, but not renal arteries.

(264 word)
INTRODUCTION

Persistent pulmonary hypertension of the newborn (PPHN) is a rapidly progressive vasculopathy, stemming from a developmentally crucial moment in normal perinatal circulatory transition. Adaptation to extrauterine life requires a complex series of structural and functional changes in the pulmonary vascular endothelium and smooth muscle, resulting in decreased pulmonary vascular resistance. PPHN constitutes a failure of normal pulmonary vascular relaxation after birth(24) due to perinatal hypoxia, inflammation or direct lung injury(14), with an incidence of up to 6 per 1000 live births(50).

Dynamic polymerization and depolymerization of actin filaments is important in the contraction-relaxation process of smooth muscle(44). Cytoskeletal disassembly is a critical component of postnatal pulmonary arterial relaxation. Postnatal reorganization of the pulmonary arterial myocyte cytoskeleton, in particular a decrease in immunoreactive alpha and gamma actin content, is important in normal circulatory transition; this structural remodeling is abrogated in pulmonary arteries from hypoxic PPHN(12). The functional role of actin polymerization in PPHN is not established.

Vasoconstrictor stimuli induce actin polymerization (APM) in smooth muscle; increased F-actin content is a key determinant of force generation(4). Thromboxane is a potent pulmonary vasoconstrictor and systemic vasodilator agent which acts via the thromboxane prostanoid receptor (TP)(51), inducing platelet aggregation, vasoconstriction and smooth muscle cell proliferation(2, 3, 13). TP, a seven transmembrane domain G-protein coupled receptor, is capable of coupling to several G-proteins; most typically Gαq/11(11) or Gα12/13(18). Coupling to Gαq/11 triggers activation of phospholipase Cγ, leading to an increase in [Ca2+]i (cytosolic free calcium) and Ca2+ sensitization of contraction(5, 15, 19, 47). In contrast, coupling to Gα12/13 leads to activation of Rho subfamily GTPases, such as Rho, Rac and Cdc42, which regulate the functional status of the actin regulator proteins (actin filament assembly or structural reorganization) and also promote Ca2+ sensitization.

RhoA and its downstream effector Rho kinase promote vasoconstriction and Ca2+ sensitivity(26, 37, 41, 49). RhoA regulates the actin cytoskeleton via the LIM kinase/cofilin pathway(25). Rho kinase-
mediated phosphorylation activates LIM kinase (LIMK), increasing phospho-cofilin(1). Phosphorylation of coflin inhibits its ability to depolymerize actin filaments(57). The resulting increase in F-actin content may contribute toward increased myocyte contractility.

In a hypoxic neonatal porcine model of PPHN, our lab previously reported TP hypersensitivity and hyperreactivity in pulmonary arterial (PA) myocytes, with persistent sensitization long after cessation of hypoxia(16, 17). We have demonstrated increased ligand sensitivity of the TP-Gαq complex, increased Ca²⁺ mobilization, altered APM, and downregulation of myosin light chain phosphatase(6). These studies established that hypoxia alters APM in neonatal PA myocytes, an effect not seen in systemic arterial myocytes. Hypoxia is known to increase both migration and proliferation of synthetic but not contractile phenotype PA myocytes. We now speculate that hypoxia modifies TP-Gαq signaling to Rho GTPases in PA myocytes, with potential impact on APM, Ca²⁺ sensitivity and muscle contraction.

The role of actin, and the mechanism regulating changes in polymerized actin content, is not well understood in myocytes of the newborn pulmonary and systemic vascular circuits. It has been unclear whether cytoskeletal remodeling in PPHN actually contributes toward increased vasoconstriction, or acts as an internal resistor opposing force generation in pulmonary arterial myocytes. In this study, we examine pathways regulating APM in an in vitro model of hypoxic pulmonary arterial and aortic myocytes exposed to the TP agonist U46619. To identify a tissue-specific role of hypoxic actin polymerization in thromboxane-induced force development, we also investigate the effects of TP stimulation on force generation and cytoskeletal assembly in resistance pulmonary and renal arteries (both of which constrict to thromboxane stimulation) from animals with hypoxic PPHN, in comparison to normoxic controls. We hypothesize [i] that hypoxia activates the RhoA pathway, increasing APM to a greater degree in agonist-naïve pulmonary arterial myocytes than in aortic myocytes; [ii] that selective coupling of TP with G-protein species is altered by hypoxia, such that APM following thromboxane challenge is favored, paradoxically, by signalling pathways other than RhoA; and [iii] that increased pulmonary arterial APM, a structural hallmark of perinatal hypoxia, specifically enhances thromboxane-induced pulmonary vasoconstriction in PPHN.
METHODS

Induction of PPHN

All protocols were approved by the University of Manitoba in accordance with the Canadian Council on Animal Care. To induce PPHN in vivo, newborn piglets (<24 hrs old; N=3) were raised in normobaric hypoxia for 72 hrs (FiO₂=0.10); PPHN was confirmed by the presence of cardiac right ventricular hypertrophy(6). Age-matched controls were raised in normoxia (FiO₂=0.21; N=3). Newborn swine (<24 hrs old; N=9) were obtained for primary myocyte culture. Piglets were euthanized by pentobarbital overdose and exsanguination. Heart, great vessels, lungs and kidneys were removed en bloc and placed in oxygenated cold (4°C) Ca²⁺-free Krebs-Henseleit buffer (containing in mM: 112.6 NaCl, 25 NaHCO₃, 1.38 NaH₂PO₄, 4.7 KCl, 2.46 MgSO₄·7H₂O, 5.56 Dextrose; pH 7.4). Resistance pulmonary and renal arteries (external diameter 300-600μm) were isolated by microdissection for myography (below).

Cell Culture and In Vitro Hypoxia

Descending aorta and 2nd to 6th generation pulmonary arteries were microdissected from newborn swine into Ca²⁺-free Krebs-Henseleit buffer. Tissues were allowed to recover in cold HEPES-buffered saline solution (HBS; composition in mM: 130 NaCl, 5 KCl, 1.2 MgCl₂, 1.5 CaCl₂, 10 HEPES, 10 glucose; pH 7.4) supplemented with antibiotic/antimycotic mixture. Pulmonary arterial and aortic smooth muscle cells were obtained using a dispersed cell culture method as previously described(40); arteries were washed twice with 20 μM CaCl₂ (Ca²⁺-reduced) HBS, finely minced, then transferred to digestion medium containing Ca²⁺-reduced HBS, type I collagenase (1750U/mL), dithiothreitol (1mM), bovine serum albumin (BSA; 2mg/mL), and papain (9.5U/mL) for 15 min at 37°C with gentle agitation. The dispersed cells were collected by centrifugation at 1200 rpm for 5 min, washed in Ca²⁺-free HBS to remove digestion
solution, then resuspended in Ham’s F-12 medium supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin and plated at a density of 4.4 x 10^4 cells/cm².

Stable sub-confluent myocyte cultures were established prior to environmental modification. Half of the culture plates were placed in a hypoxic (H) culture environment achieved by washout of a sealed incubator with a hypoxic gas mixture (7% O₂, 5% CO₂, balance N₂) to achieve a final concentration of 10±1% O₂. The remaining plates were maintained in normoxia (N; 21% O₂, 5% CO₂, balance N₂). Once serum-fed (S+) cultures reached ~75% confluence, lysates were collected from half of the plates in each condition to obtain NS+ and HS+ (synthetic phenotype) treatment groups. The remaining plates were then serum-deprived using Ham’s F-12 medium supplemented with L-glutamine/penicillin/streptomycin and 1% insulin-transferrin-selenium for 7 days, to synchronize in contractile phenotype (NS- and HS-). The four myocyte treatment groups and protocols are outlined in Fig. 1A.

Hypoxic and Thromboxane-Stimulated APM, by Laser Scanning Cytometry

Neonatal myocytes were grown under hypoxic or normoxic conditions and serum-fed or serum-deprived to produce a synthetic or contractile phenotype respectively. Half of each treatment group was stimulated for 30 min with or without 10⁻⁶M U46619 (Sigma) in media. Cells were then washed with PBS, fixed with 3% paraformaldehyde (PFA) diluted in CB buffer (containing in mM: 10 MES, 150 NaCl, 5 EGTA, 5 MgCl₂, 5 glucose), and then permeabilized with 3% PFA containing 0.3% Triton X-100. Cells were rinsed and stored in cyto-TBS buffer containing 200mM tris-base, 1.54M NaCl, 20mM EGTA and 20mM MgCl₂. Ao and PA smooth muscle cells were incubated with Alexa Fluor 633 Phalloidin (filamentous (F) actin) and DNase 488 (globular (G) actin) (Invitrogen, Molecular Probes) in cyto-TBS + 1% BSA for 1 hr at room temperature. Coverslips were washed in cyto-TBS+0.5M NaCl, and nuclei counterstained with Hoechst 33342. Laser-scanning cytometry (LSC) was used to quantify fluorescence integral intensity of F and G actin per established method(39), using a 40x objective and standardized settings of voltage, gain and offset, in defined regions surrounding nuclei. Nuclear debris and overlapping
nuclei were gated out by statistical filters. Each nucleus was then used as a primary contour to define a fixed-dimension peripheral contour extending to 7 pixels outside the nuclear perimeter, inclusive of sarcoplasm but exclusive of background. In these regions of interest (Fig. 1B-D), F and G actin staining were assessed as integral intensity of green and red fluorescence respectively, per myocyte, in agonist-naïve and U46619-challenged groups.

**Hypoxic and Thromboxane-Stimulated APM, by Cytoskeletal Fractionation and Immunoblot**

Neonatal myocytes were serum-deprived to synchronize in a contractile phenotype, then placed in either normoxic or hypoxic incubator. Cells from each treatment group were stimulated for 30 min with 10⁻⁶M U46619 in media, or diluent alone; then placed on ice and washed with PBS containing 1mM MgCl₂. Using a stress fiber isolation method adapted from Katoh et al (21), the soluble cellular components were collected over a series of 6 washes with 2.5mM triethanolamine supplemented with protease inhibitors (in μg/mL: 20 aprotinin, 1 leupeptin, and 1 pepstatin, pH 8.2), followed by lyophilization. The surface of the cells and nuclei were removed with 0.05% NP-40 and 0.5% Triton X-100 respectively. Stress fibers were then immediately washed with PBS, and collected by centrifugation at 100000xg for 1 hour. The pellet (F-actin) and lyophilized soluble components (G-actin) were resuspended in 400 μL of RIPA buffer (in mM: 20 MOPS, 2 EGTA, 5 EDTA, 30 NaF, 40 beta-glycerophosphate, 10 sodium pyrophosphate, 2 sodium orthovanadate, 1 PMSF, 3 benzamidine, 0.005 pepstatin A, 0.01 leupeptin). Equal volumes of each fraction were separated by SDS-PAGE, and the blots were probed with anti-actin (1:1000 dilution; Cytoskeleton). Protein bands were visualized by enhanced chemiluminescence and quantified under non-saturating conditions by densitometry, with manual background subtraction.

**TP-Receptor: G-protein Coupling, by Immunoprecipitation**
Neonatal myocytes were grown under hypoxic or normoxic conditions and serum-fed or serum-deprived. Half of each treatment group was stimulated for 30 min with or without $10^{-6}$M U46616 in media. Whole cell lysates were collected in RIPA buffer. Lysates (400 μg) were pre-cleared by incubation with 35μL of a 50% slurry of protein G Sepharose beads (GE Healthcare), prepared in lysis buffer (in mM: 50 Tris, 150 NaCl, 1 EDTA, and 1% Triton X-100, pH 7.4). Beads were removed by centrifugation at 16000 g for 5 min. The pre-cleared lysates were then incubated overnight at 4°C with 2 μg of rabbit-TP receptor antibody (Cayman Chemicals). The precipitate was pulled down with 30μL of 50% bead slurry. Beads were washed in lysis buffer and boiled in 4x Laemmli loading buffer. Eluted protein was separated by SDS-PAGE and transferred onto nitrocellulose membrane. Blots were probed with antisera to $G_{αq}$, $G_{12}$ and $G_{13}$ (1:400 dilution; Abcam). Protein bands were visualized by enhanced chemiluminescence (Amersham) and quantified under non-saturating conditions by densitometry, with manual background subtraction.

*Rho activity, by Affinity Precipitation*

Neonatal myocytes grown under hypoxic or normoxic conditions, as well as serum-fed and serum-deprived, were treated for 30 min with or without $10^{-6}$ M U46619. Rho activation was determined by affinity precipitation assay (Cytoskeleton). Myocyte lysates were collected in cold lysis buffer (50 mM Tris pH 7.5, 10 mM MgCl$_2$, 0.7 M NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2% Igepal) supplemented with a protease inhibitor cocktail (in μg/mL: 10 leupeptin, 10 aprotinin, and 500 tosyl arginine methyl ester). Immediately samples were sonicated and clarified by centrifugation at 10000 rpm. Active RhoA was precipitated using GST-tagged Rhotekin-RBD protein on colored agarose beads. Proteins were eluted by centrifugation and separated by SDS-PAGE. Blots were probed with anti-RhoA monoclonal antibody, and active RhoA was compared with RhoA protein abundance in total cell lysates. Addition of non-hydrolysable GTP analog GTPγS (200 μM) was used as a positive control, and excess (10 mM) GDP as a negative control.
Phosphorylation of LIMK and Cofilin, by Western Blot

Neonatal myocytes grown under hypoxic or normoxic conditions, serum-fed or serum-deprived, were stimulated for 30 min with $10^{-6}$M U46619 or diluent. Whole cell lysates were collected on ice in lysis buffer as above, and separated by SDS-PAGE. Blots were first probed with antibodies to phosphorylated forms of LIMK-1 (Millipore) and cofilin-1/2 (Cell Signaling), then stripped and re-probed for non-phosphorylated LIMK-1 (Cell Signaling) and cofilin-1/2 (Cedarlane) respectively. All antibodies were diluted 1:1000 in 5% BSA-TBST. Enhanced chemiluminescence was used to visualize protein bands. Data was quantified under non-saturating conditions, with manual background subtraction by a digital imaging densitometer.

Effect of Rho Inhibition on APM, by Immunofluorescence

Synthetic and contractile phenotype neonatal myocytes grown under hypoxic or normoxic conditions were pre-incubated in media with or without $0.5\mu$g/mL Clostridium botulinum exoenzyme C3 transferase (cell permeable inhibitor of RhoA/B/C; Cytoskeleton). Each group was stimulated for 30 min with $10^{-6}$M U46619 or diluent. Coverslips were fixed in 3% PFA, and permeabilized with 3% PFA containing 0.3% Triton X-100. Non-specific binding was blocked by incubation for 30 min at room temperature in 1% BSA+cyto-TBS. Cells were then incubated for 20 min at room temperature in a cocktail solution containing Texas Red Phalloidin and Oregon Green DNAse. Coverslips were washed in water and nuclei counterstained with Hoechst 33342 (all Invitrogen, Molecular Probes.) Digital images of F- and G-actin staining were captured by epifluorescence microscopy under standardized conditions, for agonist naïve and U46619-challenged myocytes incubated with or without C3 transferase. Overall F/G actin ratio was quantified, under non-saturating conditions, using Image-Pro software.

Force Myography, and Immunohistochemistry by Laser Scanning Cytometry
Muscular PA (300-500μm) and renal arteries (400-600μm) were microdissected and mounted on a multichamber wire myograph (Danish Myo Systems) in Krebs buffer at 37°C and pH 7.4, aerated with a 95% O₂, 5% CO₂ gas mixture. Vessels were equilibrated at optimal length (determined from a length/tension curve deriving maximum active tension to KCl stimulation) for measurement of isometric contraction to 100mM KCl, and to serial concentrations of U46619. Rings were then fixed in situ at maximum contraction for each agonist concentration (steady state, 15 min post-U46619 challenge) with 10% formalin, and embedded in OCT in an end-on orientation. 10μm cryosections were stained with Alexa-Fluor Phalloidin 633 (F-actin) and DNase 488 (G-actin) for 1 hr at room temperature, then washed in cyto-TBS+0.5M NaCl; nuclei were counterstained with Hoechst 33342. Force measurements were normalized to the cross-sectional area of each fixed vessel. F and G actin staining were quantified as red and green integral intensity respectively by LSC at 40x magnification. Within the arterial muscular layer, cells were identified by a primary contour gated to include non-overlapping nuclei. Fixed-dimension peripheral contours were then defined at 1 pixel and 7 pixels outside each primary contour, to include the sarcoplasm and exclude background; actin integral intensity was calculated for the area between these peripheral contours (Fig. 1B-D) (34).

Statistical Analysis

All data are presented as mean ± SE. Myocyte comparisons were made within each matched phenotype group (HS+ and HS- myocytes were compared with their respective NS+ and NS- controls), as well as between pre- and post-U46619 challenge groups (e.g. HS- myocytes compared to HS- stimulated with U46619), by two-way ANOVA with post-hoc Bonferroni correction, p<0.05 considered significant.

RESULTS
For the following comparisons, myocyte treatment groups are abbreviated as NS+ (normoxic serum-fed [synthetic phenotype] myocytes), NS- (normoxic serum-deprived [contractile phenotype] myocytes), HS+ (hypoxic serum-fed [synthetic phenotype] myocytes) and HS- (hypoxic serum-deprived [contractile phenotype] myocytes), derived from Ao (descending aorta) or PA (pulmonary artery). For each treatment group, smooth muscle phenotype was verified by immunostaining for myosin heavy chain, and verification of Ca\(^{2+}\) response to TP agonist U46619(36).

**Effects of Hypoxia and Thromboxane Challenge on APM in PA and Aortic Myocytes.** APM was measured by LSC following whole cell staining for F and G actin (expressed ratiometrically), and in selected conditions by immunoblot of cytoskeletal versus cytosolic fractions. Hypoxia induced a 2-fold increase in APM in S-PA myocytes. In contrast, hypoxia decreased APM slightly in S+ PA myocytes. Among hypoxic PA myocytes, phenotype was crucial; APM was markedly higher in HS- compared to HS+ (Fig. 2A). Responses of Ao myocytes were similar (Fig. 2B); hypoxia increased APM in serum-deprived cells and decreased APM in serum-fed cells. Contractile Ao myocytes in both normoxic and hypoxic conditions had a greater degree of APM than their synthetic phenotype controls.

U46619 challenge of S+ PA myocytes did not alter APM in normoxic culture, but decreased APM in hypoxia. In contrast, APM in S- PA myocytes following U46619 challenge was decreased by normoxia but increased by hypoxia (Figs. 2A and 2C). APM was decreased in Ao S+ myocytes challenged with U46619 under hypoxic conditions, while U46619 exposure significantly decreased APM in both normoxic and hypoxic S- Ao myocytes (Figs. 2B and 2C).

Immunoblot results of F/G actin in agonist-naïve and agonist-stimulated contractile PA and Ao myocytes were similar to LSC findings. Hypoxia increased APM in serum-deprived PA cells, and APM was further increased post U46619-stimulation (Fig. 2D). Contractile Ao myocytes had greater polymerization of actin under hypoxic conditions, but U46619 challenge decreased APM in both normoxic and hypoxic S-Ao myocytes (Fig. 2E).
Effects of Hypoxia and Thromboxane Challenge on TP Receptor – G protein Coupling. Association of TP with various Gα protein species was measured by co-immunoprecipitation. In contractile PA myocytes, hypoxia decreased association of Gα-protein subunits G12 and G13 with TP, and increased the co-precipitation of Gαq with TP, relative to that seen in normoxic contractile PA myocytes (Fig. 3A). In normoxic Ao myocytes, association of TP with all measured Gα protein species was lessened after serum deprivation (Fig. 3B). Hypoxia did not alter TP receptor coupling in Ao myocytes.

U46619 challenge had no effect on Gαq coupling to TP-receptor in synthetic PA myocytes, but increased co-immunoprecipitation of Gαq with TP in NS- myocytes. Association of G12 or G13 with TP was unaltered by U46619 stimulation (Fig. 4A). In Ao myocytes, U46619 exposure did not change Gαq coupling to TP receptor, but did decrease co-immunoprecipitation of G12 and G13 subunits with TP in all S+ and hypoxic S- groups (Fig. 4B). In all cases, separation and blotting of eluted proteins from beads absent immune sera (pre-cleared lysates) resulted in no visualized bands (images not shown).

Effect of Hypoxia on Basal and Thromboxane-Stimulated Rho Activity in PA and Aortic Myocytes. Rho activity in cell lysates was measured by affinity precipitation of active Rho, and normalized to total Rho protein content. Hypoxia alone did not activate Rho in HS+ PA myocytes. U46619-naïve Rho activity was low in normoxic S- PA myocytes, but was significantly elevated by hypoxia (Fig. 5A(i)). Synthetic PA myocytes had insignificant change in active Rho after U46619 challenge. Following U46619 challenge of NS- PA myocytes, Rho activity markedly increased; however HS- cells, with high basal Rho activity, demonstrated little further increase in Rho activity upon U46619-stimulation (Fig. 5A(ii)). In Ao myocytes, agonist-naïve Rho activity was unaffected by hypoxia; contractile Ao myocytes in both environmental groups had lower Rho activity (Fig. 5B(i)). Rho activity was unchanged by U46619
challenge of S+ Ao myocytes. U46619 increased Rho activity slightly in the normoxic S- group (Fig. 5B(ii)).

**Effect of Hypoxia on Thromboxane-Stimulated Phosphorylation of LIMK and Cofilin in PA and Aortic Myocytes.** Phosphorylation of downstream Rho pathway intermediates LIMK and cofilin was measured by immunoblot, normalized to total content of LIMK and cofilin. Serum deprivation itself decreased LIMK phosphorylation in normoxic PA myocytes. Hypoxia increased LIMK phosphorylation in S- PA myocytes, while decreasing p-LIMK in S+ PA myocytes (Fig. 6A(i)). Stimulation with U46619 did not further alter LIMK phosphorylation in hypoxic PA myocytes (Fig. 6A(ii)). In Ao myocytes (Fig. 6B(i)), serum deprivation increased p-LIMK in normoxic and hypoxic groups. Hypoxia decreased p-LIMK in serum-fed Ao myocytes, and in this treatment group the addition of U46619 further decreased p-LIMK (Fig. 6B(ii)).

Hypoxia increased cofilin phosphorylation in PA myocytes, while serum deprivation alone had no appreciable effect (Fig. 6C(i)). U46619 challenge increased p-cofilin in all groups (Fig. 6C(ii)). In Ao myocytes, hypoxia also increased cofilin phosphorylation, while serum deprivation had no effect (Fig. 6D(i)). U46619 exposure significantly decreased cofilin phosphorylation in most Ao treatment groups; NS-cells, in which p-cofilin was already low, did not decrease this further (Fig. 6D(ii)).

**Effects of Rho Inhibition on Hypoxic and Agonist-Induced APM.** Actin polymerization was surveyed in a semi-quantitative manner by immunofluorescence microscopy, in presence or absence of Rho pathway inhibitor C3 transferase. Effects of hypoxia on APM appear more pronounced by this method, but show similar trends when measured by digital analysis of immunofluorescence, compared to LSC methods. Only in hypoxic S- PA myocytes did Rho pathway inhibition decrease polymerization of actin (Fig. 7A). Rho inhibition increased basal APM slightly in Ao myocytes (Fig. 7B). U46619 challenge induced APM in serum-deprived PA myocytes, independent of Rho inhibition (Fig. 7C). Serum-deprived Ao myocytes decreased APM after U46619 challenge, independent of Rho inhibition (Fig. 7D).
**Contractile Force and APM in PPHN Arteries.** Force generation was measured in resistance PA and renal artery rings from 3-day-old control and pulmonary hypertensive (hypoxic) animals, by isometric wire myography. Contractile force to 100mM KCl was comparable in arteries from PPHN animals versus controls. PPHN PA exhibited markedly augmented contractile force to U46619 challenge at all doses. Renal arteries had minimal contractile responses to U46619, unaltered by PPHN (Fig. 8A).

APM was measured by LSC in arterial cross-sections fixed during maximal force development at each agonist dose. Hypoxic PPHN increased APM (F/G actin ratio) in agonist-naïve PA. U46619 challenge increased APM in a dose-dependent manner in PPHN but not in control PA (Fig. 8B). PPHN increased F-actin in unstimulated PA to a level seen in maximally-contracted PA of control animals. U46619 challenge dose-dependently decreased G-actin staining in PPHN PA (albeit without apparent increase in F-actin staining), indicating greater APM corresponding with force development. While U46619 dose-dependently increased F-actin in control PA, it also increased monomeric actin over the same dose range, resulting in no cumulative change in APM (Fig. 8C). PPHN increased APM in agonist-naïve renal arteries (Fig. 8D). PPHN renal artery fluorescence staining for F-actin increased and G-actin decreased modestly after U46619 (Fig. 8E); cumulative APM was slightly increased by TP stimulation but did not correlate closely with force generation.

**DISCUSSION**

The myocyte cytoskeleton has a dynamic structure regulating material properties and development of mechanical tension in muscle tissues. Fetal adaptation to extra-uterine life entails a rapid increase in pulmonary arterial myocyte length and surface-to-volume ratio, increasing lumen diameter and lowering resistance. These changes in cell shape are associated with rapid depolymerization of contractile and cytoskeletal filaments(14). Hypoxic PPHN prevents the transient postnatal reduction in actin content(12). A functional role for actin in pulmonary arterial hypertension is thus implied, but has never been
characterized. While it is known that polymerization of actin is important for stabilizing myofilaments and increasing force generation, there is little published data on perinatal regulation of the cytoskeleton in the pulmonary or systemic vasculature. Both hypoxia and vasoconstrictor challenge may contribute toward the failure of PA remodeling in neonatal pulmonary hypertension, by interfering with cytoskeletal reorganization. This study examines hypoxia, cell phenotype and vasoconstrictor signaling as distinct stimuli for polymerization of actin, comparing responses of systemic and pulmonary arterial myocytes to determine differing pathways regulating APM in systemic versus pulmonary circulations. It further correlates APM with thromboxane-induced force generation in PPHN arteries.

We report the novel finding that while hypoxia promotes pulmonary arterial APM via Rho-linked pathways, thromboxane-induced APM in hypoxic PA myocytes is independent of Rho. We base this conclusion in part on the evidence of a TP coupling switch in hypoxic myocytes, preferentially toward Gαq and away from G12/13. Others have suggested that Rho activity may be dually regulated by Gαq/Ca2+ and G12/13 pathways in excitatory agonist-induced contraction, as both α-adrenergic and thromboxane receptor stimulation can increase Ca2+-dependent Rho activation via Gαq(37). However under hypoxic conditions, we find RhoA is not activated by U46619. This concurs with observations that in PA myocytes, while α-adrenoceptor challenge activates Rho kinase, TP receptor stimulation does not(48). We also conclude, based on arterial myography, that the increased pulmonary arterial actin polymerization in PPHN correlates with heightened thromboxane-induced vasoconstriction.

Actin exists in cells as either filamentous (F) or globular (G) actin. Vascular smooth muscle, unlike skeletal or cardiac, contains a substantial pool of monomeric G-actin(4). G-actin spontaneously polymerizes when above its critical concentration (~8μg/mL)(35). In differentiated vascular myocytes, the cytoplasmic G-actin concentration rests above this critical threshold, requiring tight regulation by actin binding proteins such as cofilin, profilin, capping and sequestering proteins(22), to control the F:G-actin ratio and thin filament assembly or disassembly(42). Small increments in APM correspond with large increases in tension. In unstimulated contractile vascular smooth muscle, approximately 80% of total actin
exists as F-actin(56). This percentage shifts to over 90% F-actin after alpha-adrenergic stimulation(56). For the majority of experiments in this study, we measure APM as a ratio of staining intensity profiles, not by absolute separation of monomer from polymer. However these ratiometric APM measurements are validated by corresponding quantification of F- and G-actin by cytoskeletal separation and immunoblot, in selected key experimental conditions.

Actin filaments, uniquely among cell structural components, increase cell stiffness and amplify dynamic contraction force(27). Contractile agonists precipitate remodeling of the actin cytoskeleton in arteriolar myocytes, promoting tension development(31, 32). Intravascular pressure also increases APM(4, 8). Not all agonist-induced polymerization involves thin filament (smooth muscle type) α-actin; adrenergic contraction induces remodeling of γ-actin and to a lesser extent, β-actin(23). Coincidentally, the transient loss of γ-actin is implicated in neonatal hypoxic pulmonary hypertension(12). Newly polymerized actin is located submembranously, giving rise to speculation that cytoskeletal remodeling may alter the transmission of cross-bridge generated force to the cell membrane(9). Dynamic actin remodeling may also enhance the strength of connections between membrane adhesion junctions and actin filaments, providing a rigid framework for transmission of force(56). We place into this context our finding that exposure to hypoxia independently increases APM in contractile PA myocytes, thus contributing to vascular rigidity and force transmission in hypoxic vasoconstriction. Using epifluorescence laser scanning microscopy, we localize APM to a radius peripheral to each nucleus, but cannot by this method differentiate cortical from submembranous actin; that distinction may prove important when ascribing a functional role for hypoxic APM. We also cannot yet speculate as to the series or parallel nature of filament assembly under hypoxic conditions.

Agonist-naïve APM in hypoxic contractile PA myocytes appears dependent on the Rho-Rho kinase pathway. Hypoxia increases active RhoA in PA myocytes; inhibition of Rho decreases polymerized actin in HS- PA myocytes. In cultured vascular myocytes, APM during stress fiber formation(20) and receptor-mediated contraction(10) may be mediated by activation of G_{12/13} and Rho/Rho-kinase. We speculate that
the hypoxic APM measured in agonist-naïve contractile myocytes may result from paracrine vasoconstrictor receptor stimulation, activating RhoA. We observed that in PA myocytes, hypoxia causes the TP receptor to associate with Gαq, at the expense of its coupling with G12/13; we infer from this that G12/13-coupled receptors other than TP may activate Rho pathway signaling in hypoxic PA.

PA myocytes exhibit a greater range of actin plasticity than do Ao cells. This is predictable, given the structural plasticity of perinatal PA during circulatory adaptation to extraterine life. In contrast to contractile PA myocytes, hypoxia-induced APM in contractile Ao myocytes is modest, and is independent of the Rho pathway.

We examined APM in synthetic myocytes, a minority population of the vascular media, as this phenotype subpopulation tends to expand in hypoxic vessels, and its cytoskeleton is adaptive to forces of migration and proliferation(33). Both PA and Ao synthetic myocytes decrease APM during hypoxic exposure. Migratory cells are polar, requiring active remodeling of the actin cytoskeleton such that the leading end undergoes filament growth, while the lagging end requires filament disassembly. We measured cumulative changes in APM per cell, and thus cannot comment upon cell polarity. However, actin depolymerization appears to be the dominant trend. While synthetic phenotype fibroblasts require RhoA activity for cell locomotion(46), receptor-independent, protein kinase G-mediated pathways have been implicated in hypoxic pulmonary arterial myocyte adhesion, actin depolymerization and cell migration(28). In both PA and Ao synthetic myocytes, remodeling of the actin cytoskeleton is not Rho-dependent. Rho activity is unaltered by hypoxia in S+ PA and Ao myocytes, and APM is not diminished by Rho inhibition. We therefore speculate that GPCR-linked GTPases other than Rho may be important for synthetic myocyte migration under hypoxic conditions.

Thromboxane is a critical pulmonary constrictor under hypoxic conditions. We therefore modeled the effect of vasoconstrictor challenge by incubation with synthetic TP-agonist U46619. In S+ myocytes of systemic and pulmonary origins, U46619 decreases APM, suggesting TP stimulation may promote myocyte migration or proliferation. Thromboxane receptor activity is known to stimulate migration of
endothelium(7) and mesenchymal stem cells(54), and to play a role in tumor cell motility(29). In contractile PA, U46619 further increases polymerized actin, while decreasing APM in S- Ao myocytes. This finding is consistent with the fact that thromboxane is a vasoconstrictor in the pulmonary circuit, while a vasodilator in the systemic circuit. APM in U46619-stimulated hypoxic contractile PA myocytes is independent of the Rho-Rho kinase pathway, as addition of U46619 does not alter TP receptor association with Ga species, does not activate RhoA or LIMK phosphorylation, and Rho-inhibited PA myocytes still increase APM when stimulated with U46619. We conclude that APM in response to TP challenge may occur via other G12/13-linked pathways. Other candidate pathways for regulation of agonist-elicited active force and APM in smooth muscle include Cdc42(45) and the PI3K/Akt pathways(55). We are currently investigating effects of TP stimulation on APM in pulmonary artery via these pathways.

LIMK activated by Rho kinase or Cdc42 is known to cause actin stress fiber formation via cofilin phosphorylation(1, 43, 53). We observed LIMK phosphorylation to correspond closely with trends in measured APM. Phosphorylation of cofilin, a terminal effector protein which severs F-actin, suppresses cytoskeletal reorganization(25); cofilin dephosphorylation reduces agonist-induced force development(57). Unexpectedly in our hands cofilin phosphorylation corresponds less well with APM in PA. Addition of U46619 increases phosho-cofilin in normoxic and hypoxic S- PA myocytes, while APM is increased only in the hypoxic group. While regulation of cofilin is directly upstream of actin depolymerization, other factors influence cofilin activity. Cofilin phosphatases and their upstream signaling pathways in vascular smooth muscle are various. Cofilin activity may be dually regulated via LIMK and Slingshot-1L phosphatase(38); the latter intermediate is also activated downstream to phosphatidylinositol 3-kinase(30), causing LIMK-independent activation of cofilin. PI3K and Slingshot have not yet been investigated in context of hypoxic APM. It is also possible that, in examining stable APM following prolonged agonist exposure (30 min), our time course may have missed the point of maximal phosphorylation of signaling intermediates contributing to APM. In studies of vascular smooth muscle migration, cofilin was dephosphorylated anywhere between 5 and 60 minutes after stimulus(52, 55). Notwithstanding any
discrepancies in measured phospho-cofilin, the concordance of LIMK activation with APM in this study was taken to substantiate the overall pathway. Hence a time course study was not pursued.

Finally we examined actin polymerization and force responses simultaneously, in arterioles from an in vivo hypoxia model of PPHN. To isolate the tissue-specific contribution of APM to force generation, we measured responses in PA and renal arteries, both of which have a contractile response to thromboxane. Agonist-naïve PPHN pulmonary arteries, similar to hypoxic PA myocytes, have elevated F-actin content. This basal APM does not increase KCl-mediated contraction of PA; but the dose-dependent effect of TP stimulation on APM corresponds with enhanced force generation by hypoxic pulmonary artery to this agonist. Renal arteries from PPHN animals have KCl responses similar to pulmonary arteries. However while resting APM is elevated in hypoxic renal artery, there is no correlation in renal artery between agonist-induced APM and force generation to U46619. We conclude that pulmonary arterial APM is uniquely important for agonist-induced force in PPHN. Hypoxia in vivo and U46619 challenge independently increase APM in PPHN pulmonary artery, contributing toward agonist-induced vasoconstriction. These data serve to validate our in vitro observations.

In summary, hypoxia increases APM in all contractile myocytes, with the Rho pathway being particularly important in pulmonary artery. Hypoxia in vivo similarly increases pulmonary arterial APM. We speculate that actin polymerization-mediated wall stiffness and force generation in hypoxic PA may be amenable to downregulation by Rho kinase inhibitors, in which there is evolving clinical interest. However, challenge of hypoxic PA myocytes with a thromboxane mimetic induces marked APM, independently of Rho. This APM response is unique to the pulmonary circuit, facilitates agonist-mediated vasoconstriction, and has implications for therapeutic management of pulmonary arterial remodeling in the presence of inflammatory prostanoids. Receptor type, receptor coupling and concurrent activation of other pathways converging upon actin filament reorganization influence vasoconstrictor-induced APM. Inhibition of Rho pathway intermediates may not be beneficial in this context.
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REFERENCES


Figure Legends

Fig. 1. (A) *In vitro* hypoxic exposure and growth factor manipulation of PA and Ao smooth muscle cells. Myocytes were allowed to adhere for 2 days in Hams F12 media containing 10% fetal bovine serum. Culture plates were then randomly distributed into normoxia (white bars; 21% O₂) or hypoxia (black bars; 10% O₂) for 7 days. Cells were then harvested or fixed (●), or serum-deprived (F12 + 1% PS + 1% ITS) for 7 days prior to collection. For Rho inhibition study, cells were treated for 3 days with C3 transferase (♯) prior to collection. All groups were incubated with 10⁻⁶M U46619 or diluent (†), before lysis or fixation. Treatment groups consist of: synthetic (serum-fed, S+; open bars) myocytes and contractile (serum-deprived, S-; hatched bars) myocytes, cultured in normoxia (NS+ and NS-) or hypoxia (HS+ and HS-). Quantification of F and G actin in myocytes and tissue by LSC. Slides are scanned at 40x magnification to visualize blue (nuclei), red (F-actin) and green (G-actin) channels. (B) Viewing arterial sections using only the blue channel, a region of interest (R1) is selected encompassing the muscular layer. (C) Single field image from an arterial ring, illustrating primary and peripheral contours. Nuclei (20-200μm²) are selected for the primary contour (red line), and used to derive the peripheral contours (yellow lines) to calculate the integral pixel intensity of F-actin and G-actin per cell. (D) Zoomed image of a single myocyte showing F- and G-actin staining; nuclear contour and peripheral contours derived as above. The peripheral contour is expanded from the primary contour to exclude background and areas of cell overlap.

Fig. 2. Effects of hypoxia and U46619-stimulation on APM in PA and Ao myocytes, by LSC and by immunoblot. Myocytes cultured in normoxic or hypoxic conditions, serum-fed or -deprived, were stimulated with 10⁻⁶M U46619 or diluent for 30 min prior to paraformaldehyde fixation (A and B), and then stained with Phalloidin 633 (F-actin) and DNase 488 (G-actin). Nuclei counterstained with Hoechst 33342 supplied a primary contour to quantify F and G actin integral intensity per cell. (C) Representative composite LSC images of F-actin and G-actin, in agonist-naïve and U46619-challenged hypoxic or normoxic S- PA and Ao myocytes. Normoxic and hypoxic serum-deprived PA (D) and Ao (E) myocytes were stimulated with 10⁻⁶M U46619 or diluent for 30 min prior to stress fiber isolation. Stress fibers (F-actin) were isolated from the soluble components of the cells (G-actin), and separated by SDS-PAGE to quantify F/G actin ratio. (APM expressed as F/G actin ratio; N=3 piglets; ***p<0.001 compared with normoxic controls; #p<0.001 compared with their respective S- phenotype controls; ••p<0.01 and •••p<0.001 compared with U46619-naïve controls.)
Fig. 3. Effect of hypoxia on association of G-proteins to TP receptor in PA (A) and Ao (B) myocytes, measured by immunoprecipitation. Myocytes were cultured in normoxia or hypoxia, serum-fed or serum-deprived. Cell extracts were precipitated with antibody to TP, then probed with $G_{aq}$ (i), $G_{12}$ (ii) or $G_{13}$ (iii) specific antibody (Western blots shown below respective histograms; N=3 piglets; **p<0.01 compared with respective N controls; #p<0.05 compared with their respective S+ phenotype.)

Fig. 4. Effect of U46619-challenge on TP-G-protein association, by immunoprecipitation. PA (A) and Ao (B) myocytes cultured in normoxia or hypoxia, serum-fed or serum-deprived, were stimulated with 10^-6M U46619 or diluent for 30 min prior to lysis. Cell extracts were precipitated with TP-specific antibody and then probed with antibody to $G_{aq}$ (i), $G_{12}$ (ii) or $G_{13}$ (iii) (Western blots shown below respective histograms; N=3 piglets; *p<0.05 and **p<0.01 compared with U46619-naïve controls.)

Fig. 5. Rho activation in agonist-naïve and U46619-challenged PA (A) and Ao (B) myocytes, by affinity precipitation. Myocytes cultured in normoxic and hypoxic conditions, serum-fed or -deprived, then stimulated with 10^-6M U46619 for 30 min before lysis. Cell extracts were precipitated with GST-tagged Rhotekin-RBD protein. Rho activity expressed as ratio of active:total Rho abundance, in agonist-naïve (i) and U46619-challenged myocytes (ii). Representative Western blots shown in (iii). (N= 3 piglets; (i): *p<0.05 compared with respective N controls; #p<0.01 compared with respective S+ phenotype; (ii): *p<0.05 and **p<0.01 compared with U46619-naive controls.)

Fig. 6. Basal and U46619-stimulated activation of LIMK1 and cofolin-1/2, measured in PA (A and C) and Ao (B and D) myocytes grown in normoxia or hypoxia, serum-fed or -deprived, and challenged with 10^-6M U46619 or diluent for 30 min prior to lysis. Whole cell lysates were separated by SDS-PAGE, and blots probed with antibodies to LIMK1, phospho-LIMK, cofolin-1/2 and phospho-cofilin in agonist-naïve (i) and U46619-stimulated (ii) PA and Ao myocytes. Representative Western blots shown in part (iii). (N=3 piglets; (i) *p<0.05, **p<0.01 and ***p<0.001 compared with respective N controls; #p<0.05 compared with their respective S+ phenotype, (ii) *p<0.05, **p<0.01 and ***p<0.001 compared with U46619-naive controls.)

Fig. 7. Effect of Rho inhibition on hypoxia-induced APM in PA (A) and Ao (B) myocytes; and on U46619-induced APM in PA (C) and Ao (D) myocytes, measured by epifluorescence microscopy. Myocytes were cultured in normoxia and hypoxia, serum-fed or -deprived, and pre-incubated for 3 days in media with or without 0.5μg/mL C3 transferase; then challenged with 10^-6M U46619 or diluent for 30 min
prior to paraformaldehyde fixation. Myocytes were stained with Texas Red Phalloidin (F-actin) and Oregon Green DNase (G-actin). APM expressed as semi-quantitative F/G actin ratio; note differing y-axis scales between PA and Ao histograms. (N=3 piglets; A and B: **p<0.01 and ***p<0.001 compared with untreated C3 transferase controls; C and D: **p<0.01 and ***p<0.001 compared with U46619-naïve controls.)

**Fig. 8.** Isometric contractions to 100mM KCl, and to serial concentrations of U46619, measured in 3-day-old Control and PPHN resistance PA and renal arteries (A). Rings fixed at maximum contraction (15 min post-U46619 challenge) were cross-sectioned and stained with Phalloidin 633 (F-actin) and DNase 488 (G-actin) for laser-scanning cytometry. Nuclei were counterstained with Hoechst 33342 to define primary contours, and thence derive peripheral contours to quantify F- and G-actin integral pixel intensity within the muscular layer of pulmonary (C) and renal (E) arteries (expressed relative to mean intensity of F-actin in Control agonist-naïve pulmonary or renal artery respectively). Composite F/G actin ratio was calculated from absolute integral pixel intensities, to quantify APM in PA (B) and renal arteries (D). (N=3 piglets; *p<0.01 and **p<0.001 compared with Control arteries.)
Fig 1: Methods for Environmental Oxygen and Growth Factor Manipulation in Culture and for Quantifying F/G actin ratio in Myocytes and Tissue by LSC
Fig 2: Effects of Hypoxia and TP Agonist Challenge on APM in PA and Ao Myocytes
Fig 3: Effects of Hypoxia on G-protein Coupling to TP in Agonist-Naïve PA and Ao Myocytes

A (i)  
**PA: Gαq**

![Bar chart showing optical density for Gαq in PA with NS+, HS+, NS-, HS- conditions.](image)

(ii)  
**PA: G12**

![Bar chart showing optical density for G12 in PA with NS+, HS+, NS-, HS- conditions.](image)

(iii)  
**PA: G13**

![Bar chart showing optical density for G13 in PA with NS+, HS+, NS-, HS- conditions.](image)

B (i)  
**Aorta: Gαq**

![Bar chart showing optical density for Gαq in Aorta with NS+, HS+, NS-, HS- conditions.](image)

(ii)  
**Aorta: G12**

![Bar chart showing optical density for G12 in Aorta with NS+, HS+, NS-, HS- conditions.](image)

(iii)  
**Aorta: G13**

![Bar chart showing optical density for G13 in Aorta with NS+, HS+, NS-, HS- conditions.](image)
Fig 4: Effects of TP Agonist Challenge on G-protein Coupling in PA and Ao Myocytes

A (i) **PA: Gαq**

B (i) **Aorta: Gαq**

A (ii) **PA: G12**

B (ii) **Aorta: G12**

A (iii) **PA: G13**

B (iii) **Aorta: G13**
Fig 5: Basal and U46619-Stimulated Rho Activation in PA and Ao Myocytes

A (i)

PA

GTP-RhoA / Total RhoA

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B (i)

Aorta

GTP-RhoA / Total RhoA

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A (ii)

PA

GTP-RhoA / Total RhoA

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B (ii)

Aorta

GTP-RhoA / Total RhoA

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A (iii)

PA

GTP-RhoA / Total RhoA

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B (iii)

Aorta

GTP-RhoA / Total RhoA

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Fig 6: Basal and U46619-Stimulated Phosphorylation of LIMK and Cofilin in PA and Ao Myocytes

A (i)

B (i)

(iii)

(iii)

C (i)

D (i)

(iii)

(iii)
Fig 7: Effects of Rho Inhibition on APM in Basal and U46619-stimulated PA and Aortic Myocytes
Fig 8: Effects of TP Agonist Challenge on Contractile Force and APM in Pulmonary and Renal Arteries from PPHN and Control Animals

A
Maximal KCl Response

B
PA

C
Control PA: F-actin

D
Renal

E
Control Renal: F-actin

F
Control Renal: G-actin

G
PPHN Renal: F-actin

H
PPHN Renal: G-actin