Cell contractility and Focal Adhesion Kinase control circumferential arterial stiffness

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ABSTRACT

Arterial stiffening is a hallmark of aging and cardiovascular disease. While it is well established that vascular smooth muscle cells (SMCs) contribute to arterial stiffness by synthesizing and remodeling the arterial extracellular matrix, the direct contributions of SMC contractility and mechanosensors to arterial stiffness, and particularly the arterial response to pressure, remain less well understood despite being a long-standing question of biomedical importance. Here, we have examined this issue by combining use of pressure myography of intact carotid arteries, pharmacologic inhibition of contractility, and genetic deletion of SMC focal adhesion kinase (FAK). Biaxial inflation-extension tests performed at physiological pressures showed that acute inhibition of cell contractility with blebbistatin or EGTA altered vessel geometry and preferentially reduced circumferential, as opposed to axial, arterial stiffness in wild-type mice. Similarly, genetic deletion of SMC FAK, which attenuated arterial contraction to KCl, reduced vessel wall thickness and circumferential arterial stiffness in response to pressure while having minimal effect on axial mechanics. Moreover, these effects of FAK deletion were lost by treating arteries with blebbistatin or by inhibiting myosin light chain kinase. The expression of arterial fibrillar collagens, the integrity of arterial elastin, or markers of SMC differentiation were not affected by deletion of SMC FAK. Our results connect cell contractility and SMC FAK to the regulation of arterial wall thickness and directionally-specific arterial stiffening.
INTRODUCTION

The arterial system is composed of large, elastic arteries such as the aorta and carotids and smaller muscular arteries (1–4). The large arteries undergo periodic cycles of contraction and relaxation, which helps to dampen changes in arterial pressure during the cardiac cycle. These arteries contain a thin layer of endothelial cells called the "intima" that provides a non-thrombogenic surface around the lumen, a thick "media" comprised of vascular smooth muscle cells (SMCs) that mediate periodic arterial contraction, and a relatively diffuse outer layer called the "adventitia" that contains fibroblasts as well as inflammatory and precursor cells. Large arteries also contain an extensive extracellular matrix (ECM), and the two best studied arterial ECM components, elastin and fibrillar collagens, have important and distinguishable roles in arterial mechanics. Elastin allows for arterial recoil and dominates arterial stiffness at low stress and stretch while arterial collagens (mostly collagen-I) drive arterial stiffness in response to higher stress and stretch.

Arterial stiffening with aging and vascular disease has often been viewed as a consequence of ECM remodeling (typically, increased abundance of fibrillar collagens and/or degradation of elastin (2,3)). However, arterial stiffness is better conceptualized as the combined contributions of "material" and "functional" (also called "structural") stiffness (3,5). Material stiffness is thought to reflect the contribution of the arterial ECM while functional stiffness incorporates vessel geometry (diameter, wall thickness, etc.) as well as material stiffness (3,5).

Arterial stiffness can be studied in vivo by pulse wave velocity, but this approach tends to provide less mechanical insight than can be obtained by ex vivo methods. Pulling on isolated aortic rings or indenting on the luminal surface of isolated arterial (e.g. with an atomic force microscope) have been mainstay approaches. However, these procedures are performed in the absence of arterial pressure. This limitation can be addressed by performing inflation-extension tests on a pressure myograph, which not only measures the arterial response to physiological or pathological pressures but can also distinguish directionally specific arterial stiffening (5,6). Using
this approach, we and others have shown that age-dependent stiffening occurs in both the axial
and circumferential directions (7–10).

Recent work with the large arteries of genetically altered mice suggest that these bi-
directional changes in arterial mechanics may be differentially regulated. For example, we
reported that deletion of matrix metalloproteinase-12 (MMP12) preferential reduces axial
stiffening (7) while expression of Progerin (a mutant LaminA causative for Hutchinson-Guilford
Progeria Syndrome) increases the abundance of medial lysyl oxidase and preferentially affects
circumferential stiffening, at least early in disease progression (8). Deletion of fibulin-5 alters axial
stretch (11). Each of these genes (MMP12, lysyl oxidase and fibulin-5) has an established role in
elastin or collagen biology, supporting the important role of the ECM in arterial stiffness.

Medial SMCs (and adventitial fibroblasts) synthesize the ECM components and ECM-
modifying enzymes that play an important role in arterial stiffness. However, the degree to which
SMCs make and secrete ECM components is thought to depend on their differentiation state (12–
14). Healthy vascular SMCs exist in a differentiated state often called "contractile." These cells
are relatively quiescent and express high levels of smooth muscle contractile proteins (also called
differentiation genes or differentiation markers) such as smooth muscle-myosin heavy chain (SM-
SMC) and smooth muscle actin (SMA). The expression of SMC differentiation genes is
canonically regulated by the SRF/MRTF pathway, which in turn is downstream of Rho and its
effector Rho kinase (15,16). At sites of injury or inflammation, contractile vascular SMCs can de-
differentiate into a state often called "synthetic." A popular current model is that arterial stiffening
is a consequence of SMC de-differentiation to a synthetic phenotype because de-differentiated
SMCs have a reduced expression of contractile proteins and an increased ability to synthesize
ECM components and ECM remodeling enzymes. Indeed, de-differentiated SMCs appear at sites
of injury and inflammation coincident with arterial stiffening in vivo (17–19).
Several investigators have postulated that, in addition to the ECM, the intrinsic contractility of vascular SMCs themselves also contributes to large artery stiffness, particularly in aging and hypertension (20–23). For example, using atomic force microscopy, others have reported that the SMCs isolated from spontaneously hypertensive rats are stiffer than SMCs isolated from normotensive controls and that this difference is dependent on actin polymerization and the Rho-Rho kinase-myosin light chain kinase (MLCK) contractility pathway (21,23–25). Other work has uniaxially stretched artificial arteries generated with SMCs from hypertensive or aged animals. These fabricated "arteries" were stiffer than those containing controls SMCs, and this difference was again dependent on actin polymerization and contractility (21,23). Morgan and colleagues, using uniaxial stretch of mouse aortic segments, reported that tissue stiffness is increased in response to alpha 1-adrenergic receptor activation: in young mice, this stiffening is sensitive to pharmacologic inhibition of MLCK, non-muscle myosin II, focal adhesion kinase (FAK), and Src (20,26,27). Direct inhibition of myosin motors with blebbistatin reduced the Src-dependent phosphorylation of FAK and paxillin (27). However, Humphrey and colleagues have reported that medial and especially adventitial collagen levels can increase in chronic hypertensive and non-hypertensive mouse models showing a reduced arterial contractile capacity (28,29).

While the results outlined above have implicated SMC contractility in arterial stiffness, they have relied on mechanical analyses of isolated SMCs or ex vivo approaches that are performed independent of biologically relevant pressure. Here, we have studied the role of cell contractility in carotid artery stiffness by combining biaxial inflation-extension tests on a pressure myograph with rapid pharmacologic inhibition of myosin motors or MLCK, calcium chelation, and genetic deletion of FAK from SMCs. Since FAK deletion is a chronic approach, we also looked for effects of FAK deletion on the arterial ECM and SMC differentiation. Our results support and extend prior studies supporting a direct role for SMC contractility in large artery stiffness. Additionally, they reveal that SMC FAK contributes to the contractility effect independent of changes in fibrillar
collagens or SMC differentiation and that both cell contractility and SMC FAK selectively target circumferential arterial mechanics by regulating vessel geometry.

MATERIALS AND METHODS

Mice and artery isolation

Floxed FAK mice were obtained from the Mutant Mouse Repository and Research Center (MMRRC, stock number 009967-UCD, backcrossed to the C57BL/6J background for at least 10 generations, mated to a tamoxifen-inducible smooth muscle-specific Cre line ((30) also backcrossed to C57BL/6J), treated with ethanol (vehicle) or tamoxifen (Sigma Aldrich) for 5 days as described (31,32), allowed to recover for 1-2 weeks, and analyzed at 15-21 weeks of age. Male wild-type (WT) mice (C57BL/6J) were purchased from Jackson Laboratories and analyzed at 13-15 weeks of age. Mice were fed a chow diet ad libitum. Mice were sacrificed with CO₂, and the right carotid artery was stripped of most fat in vivo, removed, and then briefly stored in Hanks buffered Salt Solution (HBSS) with magnesium and calcium without phenol red (Corning Life Sciences) prior to myography. The mice were then perfused with PBS through the left ventricle, and the left carotid artery was removed and fixed in Prefer for paraffin-embedding and tissue immunostaining. Because the Cre transgene is integrated into the Y chromosome (30), all experiments were performed on male mice. Animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Carotid artery immunostaining and histologic analysis. The immunofluorescence analysis of isolated carotid arteries for ECM proteins and differentiation markers was performed largely as described (8,33) and used the following antibodies: collagen-I (Southern Biotech, 1310-01; 1:300 dilution), collagen-III (Proteintech, 22734-1-AP; 1:300 dilution), collagen-V (Abcam, ab7046; 1:250 dilution), SM-MHC (Protein Tech 21404-1-AP, diluted 1:200 in PBS) or SMA (FITC-conjugated; Sigma F3777, diluted 1:300 in PBS). FAK levels were determined using a primary
FAK antibody (BD Transduction Laboratory; 610088) diluted 50-fold in 1% goat serum in PBS and then incubated for 2 hr at 37°C followed by overnight incubation at room temperature. The secondary antibody for FAK (Alexa Fluor 594 goat anti-mouse IgG; Invitrogen) was used at a 100-fold dilution in 1% goat serum in PBS and incubated with the samples for 2 hr at room temperature. All samples were mounted with Dapi Fluoromount-G (Southernbiotech). Results were visualized with a Nikon Eclipse 80i microscope with a QI-Click Qimaging camera. Carotid arteries were imaged at 20x magnification.

Images were quantified using Fiji. The media of each section was traced using the polygon drawing tool, and its raw integrated intensity was divided by the area of the outlined media to obtain relative fluorescence intensity. In some experiments, this procedure was repeated for the adventitial layer.

Analysis of arterial mechanics by pressure myography

Passive arterial mechanics. Axial and circumferential mechanics were determined using a DMT 114P pressure myography with force transducer largely as described (7). Right carotid arteries were isolated from male C57BL/6J mice or male FAKff;iCre mice, cleaned, secured to 380 µm (outer diameter) cannulas using silk sutures, and submerged in 5 ml of calcium-containing Hanks Balanced Salt Solution (HBSS). This closed system was checked for leaks by pressurizing the vessel to 30 mm Hg using HBSS. The arteries were also visualized by light microscopy, and any remaining fat that could affect imaging was removed. The unloaded/unpressurized axial length was measured when the artery transitioned from being bent to straight, stretched to 1.7 of that length, and pressurized to 100 mm Hg for 15 min in calcium-containing HBSS. The arteries were preconditioned by cyclic pressurization three times from 0–140 mm Hg in 1-min increments. Unloaded (unstretched and unpressurized) vessel wall thickness and outer diameter were measured in multiple sections after preconditioning and averaged for post-test data analysis (see Table S1).
In vivo stretch (IVS) was determined using force-length tests largely as described (5,7) except that the carotid arteries were axially stretched at four constant pressures (80, 100, 120, 140 mm Hg). Equilibrium force was recorded for each stretch and pressure, and the intersection of force-stretch curves defined the IVS. Loaded inner radius and wall thickness were determined from pressure-outer diameter tests with samples at their IVS and pressurized in 10-mm Hg (30-sec) steps from 0-140 mm Hg before returning the artery to 0 mm Hg. We confirmed the validity of our IVS determinations by measuring axial force through the circumferential tests, and we excluded samples where axial force with pressure varied from the mean by >25%.

Raw measurements of the intraluminal pressure, force transducer readings, and video-tracked outer diameter were converted into stress-stretch curves using equations 1–4.

Equation 1: Axial stretch \( \lambda_z = \frac{l}{L} \)

Equation 2: Axial stress \( \sigma_z = \frac{Pa^2\pi + f_T}{nh(2a + h)} \)

Equation 3: Circumferential stretch \( \lambda_\theta = \frac{a + h/2}{A + H/2} \)

Equation 4: Circumferential stress \( \sigma_\theta = \frac{Pa}{h} \)

where \( l \) and \( L \)=loaded and unloaded vessel lengths, respectively (µm), \( a \) and \( A \)=loaded and unloaded inner radii, respectively (µm), \( h \) and \( H \)=loaded and unloaded vessel wall thickness, respectively (µm), \( P \)=intraluminal pressure (mm Hg), and \( f_T \)=axial force (nN).

Vessel wall thickness was calculated in the post-test analysis based on the sample being incompressible (see (7)). Each axial and circumferential test was performed once; results were accrued from multiple mice and plotted as mean ± SD.

Effects of FAK, blebbistatin, EGTA and ML7 on passive mechanics. Isolated carotid arteries from WT male mice or the male FAK\( ^{ff} \);iCre mice treated with ethanol or tamoxifen were cleaned of excess fat, mounted on the DMT 114P pressure myograph and incubated in 5 ml HBSS as
described above. To examine the effect of blebbistatin (EMD Millipore), EGTA and ML7 (Cayman Chemical) on passive arterial mechanics, each carotid artery of the WT and FAK$^{ff};i$Cre mice that had been subjected to passive testing was immediately de-pressurized, and then brought to 100 mm Hg and then treated with the relevant reagent (10 $\mu$M blebbistatin or 2 mM EGTA for WT mice; 10 $\mu$M blebbistatin or 20 $\mu$M ML7 for FAK-deficient mice) by replacing the HBSS bath with 5 ml of reagent-containing HBSS. The complete set of axial and circumferential tests was repeated after maximum vessel dilation had occurred in response to each reagent (5-10 min). The effect of each drug was monitored by the increase in outer diameter (in right carotid arteries at 100 mm Hg). Mean ± SD changes were 2.4% ± 1.7 (blebbistatin), 2.7% ± 1.8 (ML7) and 10.1% ± 3.2 (EGTA).

**Statistical Analysis.** Statistical analyses were performed using Prism software (Graphpad). Differences in carotid artery immunofluorescence staining were analyzed using 2-tailed Mann-Whitney tests. For the myography experiments, differences in IVS were analyzed by 2-tailed t-tests. The significance of changes in the pressure-outer diameter curves, stress-stretch curves, pressure-inner radius curves, pressure-wall thickness curves were assessed using two-way ANOVAs. The statistical tests used are identified in the respective figure legends; significance is indicated by *(p<0.05), **(p<0.01), ****(p<0.001) and *****(p<0.0001).

**RESULTS**

**Acute pharmacologic inhibition of carotid artery contraction selectively reduces circumferential arterial stiffness in WT mice**

We began our studies on the role of cell contractility in arterial stiffness by acutely treating freshly isolated carotid arteries from WT mice with blebbistatin (a well-established inhibitor of myosin II-mediated contractility) and then determining the consequence for arterial geometry and
stiffness. Treatment times were very short (~ 5 min), eliminating the likelihood of long-term changes in cell differentiation or ECM composition that could confound the analysis.

Arterial mechanics were analyzed with biaxial inflation-extension tests performed at biologically relevant pressures. Each carotid artery was analyzed twice, first in the absence and then presence of blebbistatin. We could hardly detect an effect of blebbistatin on axial mechanics as determined by the IVS (Fig. 1A) or the axial stretch-strain relationship (Figs. 1B and S1A-C). However, we detected clear effects of blebbistatin on the circumferential arterial response to increasing pressure. In particular, arterial outer diameters (Fig. 1C) and inner radius measurements (Fig. 1D) were increased in the blebbistatin-treated arteries as compared to the WT controls. The blebbistatin-treated arteries also had a significantly reduced wall thickness (Fig. 1E) in response to pressure. These effects were associated with reduced circumferential stiffness as shown by the overall right-shift in the stretch-strain curve (Fig. 1F). We note, however, an increase in the slope of the stretch-stress curve for blebbistatin-treated arteries at circumferential stretches >2.

As calcium is critical to SMC contractility (34,35), we tested the requirement for calcium by performing the entire set of passive testing of WT carotid arteries in the absence versus presence of the calcium chelator, EGTA (Fig. 2). The effects of EGTA were very similar to those seen with blebbistatin, with the primary effect being on circumferential (Fig. 2F) rather than axial arterial stiffness (Fig. 2B). We note, however, that EGTA did reduce axial stiffness at 80 mm Hg (but not 100, 120 or 140 mm Hg; Figs. 2B and S1D-F). The basis for this isolated effect is not clear but may relate to the fact that calcium chelation with EGTA can also disrupt cell-cell adhesion through cadherins and cell-ECM adhesion through certain integrins (36–39).

**Smooth muscle-specific deletion of FAK affects vessel wall thickness and reduces circumferential arterial stiffness**
Our finding that cell contraction contributes to arterial geometry and circumferential arterial stiffness led us to postulate that upstream regulators of contractility should play a role in this effect. We were particularly interested in exploring the role of FAK because it is a canonical mechanosensor that transduces signals from multiple ECM-integrins complexes, because its activating phosphorylation in SMCs is stimulated by ECM stiffness (31,32) and because it has been reported to promote smooth muscle cell contractility and phenylephrine-induced arterial stiffening (26,40,41).

We generated floxed FAK mice harboring a SMC-specific, tamoxifen-inducible Cre transgene (hereafter called FAKflo;ICre mice), treated the mice with vehicle (ethanol) or tamoxifen, and studied the role of SMC FAK on arterial biology. FAK expression was strongly expressed in the carotid medial layer of mice treated with vehicle and strongly reduced in the tamoxifen-treated mice (Figs. 3A-B). In contrast and as expected from the high specificity of the Cre transgene (30), adventitial FAK abundance was not significantly affected by tamoxifen. (Figs. 3A-B). Carotid artery morphology, as determined by H&E staining, appeared normal in both the vehicle and tamoxifen-treated mice (Fig. 3A).

We then performed biaxial inflation-extension tests to assess the effects of FAK deletion on arterial mechanics. Like the blebbistatin or EGTA treatments, axial arterial mechanics, as determined by the IVS and axial stretch-strain relationship, were not affected by the deletion of SMC FAK (Figs. 4A-B and S1G-I). Somewhat unexpectedly, the outer diameter (Fig. 3C) and inner radius (Fig. 4D) responses to pressure were also not affected by the deletion of SMC FAK. However, wall thickness was reduced by deletion of SMC FAK (Fig. 4E), as was circumferential arterial stiffness as determined by the right-shift in the stretch-strain curves (Fig. 4F). Thus, deletion of SMC FAK recapitulated some but not all of the circumferential effects seen with blebbistatin or EGTA.
Reduced circumferential arterial stiffness in FAK-deficient mice occurs without changes in fibrillar collagens or smooth muscle differentiation markers

Because Cre-mediated excision of FAK occurred over 2-3 weeks (see Methods), we considered the possibility that the reduced circumferential stiffness of FAK-deficient carotid arteries might reflect remodeling of the arterial ECM, and particularly a change in the abundance of fibrillar collagens or integrity of elastin, the ECM components most commonly considered to regulate ECM mechanics. However, the abundance of all three arterial fibrillar collagens (I, III and V) as well as their expected preferential localizations to the adventitia (collagen-I) or media (collagen-III and -V) were similar in the vehicle- and tamoxifen-treated carotid arteries (Figs. 5A-B). Elastin integrity was also unaffected by tamoxifen treatment (Fig. S2).

We also considered the possibility that FAK deletion might promote the transition of arterial smooth muscle cells towards a de-differentiated phenotype. However, the expression of SM-MHC and SMA proteins, two well established markers of the contractile smooth muscle phenotype (see Introduction), was similar in the carotid arteries of the vehicle- and tamoxifen-treated mice (Figs. 6A-B). As de-differentiated SMCs are thought to be responsible for ECM remodeling, the lack of a notable FAK effect on SMC differentiation supports and extends our results with the arterial collagens. Collectively, these results argue that the reduced circumferential arterial stiffness we observe upon deletion of SMC FAK is not due to a remodeled ECM arising from an altered SMC differentiation phenotype.

Acute inhibition of cell contractility attenuates the effect of FAK deletion on wall thickness and circumferential arterial stiffness

Given that FAK depletion from SMCs did not strongly affect SMC differentiation markers or the arterial collagens and elastin, we tested whether deletion of SMC FAK reduced arterial contractility. Consistent with results by others (26,40,41), we found that arterial contraction to KCl was reduced in the carotid arteries lacking SMC FAK (Fig. 7). We then eliminated smooth muscle
contraction as a possible FAK target by exposing the vehicle- and tamoxifen-treated carotids from FAK^ff;iCre mice to blebbistatin and asked if the presence or absence of SMC FAK would still affect arterial mechanics. We used blebbistatin in this analysis rather than EGTA to avoid potential confounding effects of disrupted cell-cell and/or cell-ECM adhesion. The presence or absence of SMC FAK did not significantly affect axial mechanics in the blebbistatin-treated arteries as determined by the IVS or axial stretch-strain relationship (Figs. 8A-B and S1J-L). Similarly, the outer diameter and inner radius responses to increasing pressure in the blebbistatin-treated arteries remained unaffected by the presence or absence of SMC FAK (Figs. 8C-D). However, blebbistatin treatment eliminated the ability of FAK to regulate wall thickness in response to pressure (Fig. 8E), and this effect was associated with near complete loss of the FAK effect on circumferential arterial stiffness (Fig. 8F). These results causally link the effects of FAK on smooth muscle contractility to the FAK effects on arterial geometry and stiffness. Note that the outer diameter, inner radius, wall thickness and circumferential stretch-strain relationship in the carotids of these conditional FAK mice remained sensitive to blebbistatin (Fig, S3), much like that seen in the WT vessels. Finally, we repeated the entire panel of passive mechanical testing on FAK-containing and FAK-deficient arteries treated with the myosin light chain kinase inhibitor, ML7 (Fig. S4). Consistent with the results above, deletion of SMC FAK did not affect the metrics of axial stiffness (Figs. S4A-E; compare EtOH versus Tamox solid lines), but it reduced arterial wall thickness (Fig. S4H; compare EtOH versus Tamox solid lines) and circumferential stiffness (S4I; EtOH versus Tamox solid lines). As with blebbistatin, the effects of SMC FAK deletion were lost when the arteries were treated with ML7 (Figs. S4H and S4I; EtOH versus Tamox dashed lines).

DISCUSSION

The work presented here examines the direct role of SMCs and cellular contraction in large artery stiffness. Prior work in this area has provided provocative results (see Introduction)
but has not incorporated the role of arterial pressure, interrogated possible anisotropic effects of cell contractility, or identified molecular regulators genetically. Our work addresses these limitations, and our findings indicate that i) contractility plays a clear role in the large artery stiffness response to pressure, ii) SMC FAK contributes to this effect, and iii) the consequence of cell contractility and SMC FAK on arterial stiffness is anisotropic (circumferential) and due to changes in vessel geometry. FAK deletion did not phenocopy all the geometric effects seen upon inhibition of cell contractility with blebbistatin or EGTA, but the reductions in wall thickness seen with reduced contractility (whether thought drug treatment or FAK deletion) were very similar. We have previously described a direct relationship between traction force and cell area in SMCs (42), indicating that the reductions in SMC contractility and arterial wall thickness observed in this report may be related.

While the blebbistatin, EGTA, and ML7 effects on arterial geometry and circumferential stiffness were rapid, the conditional deletion of SMC FAK occurred over 2-3 weeks. The protracted nature of this genetic deletion raised an alternative possibility that the observed FAK effect on wall thickness and the circumferential stretch-strain relationship might reflect a change in SMC differentiation rather than a direct effect on SMC contractility. However, the expression levels of SM-MHC and SMA, the two best studied SMC differentiation markers, were not affected by FAK deletion. Moreover, ECM synthesis is much more pronounced in de-differentiated (synthetic) vs. differentiated (contractile) SMCs, but we found similar fibrillar collagen levels in FAK-expressing and FAK-deleted carotid arteries under the condition of our analysis. Finally, the effect of FAK deletion on vessel wall thickness and the circumferential stretch-strain relationship was lost when contractility was blocked by acute blebbistatin treatment.

In prior work using cells cultured on deformable substrata and in vivo vascular injury, we identified a stiffness-stimulated signaling module that is comprised of FAK, Src, and p130Cas and leads to the stiffness-dependent activation of Rac (31,32). It is now interesting to view this prior work in the context of studies from Morgan and colleagues who reported activating
phosphorylations of FAK and Cas during phenylephrine-induced arterial stiffening (26,27). Both sets of studies indicate that the FAK-Src-Cas signaling module is stiffness-regulated and also describe a requirement for actomyosin contraction to maintain this signaling module in an active state. We therefore speculate that Rac activity may be upregulated in stiffened arteries, and it might be interesting to look for changes in Rac effectors in stretched aortic rings or pressure-induced extension of carotid arteries.

The Rho/Rho kinase pathway is an established regulator of SMC contractility (see Introduction), and Rho signaling is activated by both cadherin-mediated cell-cell adhesion and integrin-mediated cell-ECM adhesion (43). Our current experiments using EGTA, blebbistatin, ML7 and SMC FAK deficiency cannot resolve the relative contributions of different upstream contractility regulators and pathways to arterial stiffness. We have started to investigate this issue by generating and testing mice conditionally deficient for SMC N-cadherin, and we suggest that a similar conditional knock-out approach of putative contractility regulators (rather than use of systemic pharmacologic reagents) will be increasingly important in future studies as it allows for selective targeting of medial SMCs, intimal endothelial cells, and adventitial fibroblasts.

We note that blebbistatin, EGTA, ML7 and SMC FAK deletion do not have identical effects on circumferential arterial responses to pressure. For example, all the treatments reduced wall thickness but SMC FAK deletion did not alter the inner radius response to pressure. Thus, the inner radius response to pressure is independent of SMC FAK. One possible explanation for this data is that a different SMC focal adhesion mechanosensor regulates the inner radius response that is seen upon pharmacologic inhibition of contractility. Alternatively, the different effects of blebbistatin, EGTA, and ML7 versus SMC FAK deficiency on vessel geometry may reflect a systemic effect of the drugs on adventitial fibroblast or intimal endothelial myosin-II as compared to the tissue-specific effect of SMC FAK deletion. We also note that blebbistatin, EGTA and ML7 (but not SMC FAK deletion) reduced axial stiffness to varying degrees at 80 mm Hg but not at 100, 120 or 140 mmHg. This limited axial effect might also reflect systemic effects of the drugs.
on vessel endothelial cells and/or fibroblasts. Finally, we note that the effects of ML7 on vessel
outer diameter, inner radius, and circumferential stiffness were less pronounced than those seen
with blebbistatin or EGTA. Perhaps this difference relates to different efficiencies of drug uptake
into the carotid artery or different efficiencies of contraction inhibition. Additional tissue-specific
knock-outs as discussed above would help to resolve these possibilities.

Our results reveal an anisotropic effect of cell contractility and SMC FAK on circumferential
arterial stiffness, and it is tempting to speculate on how this might occur. One possibility relates
to the fact that SMCs (2,44) and their actin stress fibers (44) are mostly oriented circumferentially
within the arterial medial layer. Since FAK is contained within the focal adhesions that anchor
these circumferentially oriented stress fibers, reduced SMC contractility upon FAK deletion might
target circumferential mechanics more than axial. Unraveling how SMC contractility, FAK, and
other focal adhesion proteins might cooperate with ECM remodeling/integrin signaling and cell-
cell adhesion to determine directionally-selective arterial stiffening is likely to provide valuable
insight into the dynamic changes in arterial mechanics that occur with vascular development, age,
and disease.

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AUTHOR CONTRIBUTION STATEMENT
Experiments were designed by RKA and performed by ER and TX. ER, TX and RKA analyzed
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Conflict of interest statement
The authors declare that there is no conflict of interest that could be perceived as prejudicing
the impartiality of the research reported.
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FIGURE LEGENDS

Figure 1. Reduced smooth muscle contraction with blebbistatin selectively affects circumferential arterial mechanics in WT carotid arteries. Right carotid arteries isolated from male WT (C57BL/6J) mice (n=6) were analyzed by pressure myography and then immediately reanalyzed after treatment with blebbistatin (see Methods). (A) In vitro stretch as determined from axial force-length tests. (B) Axial stretch-stress curves obtained at 100 mm Hg. (C) Changes in outer diameter with increasing pressure. (D-E) Changes in vessel inner radius and wall thickness, respectively, with pressure. (F) Circumferential stretch-stress curves with data points showing results at 0-140 mm Hg in increments of 10 mm. Statistical significance was determined by 2-tailed t-test (panel A) or 2-way ANOVA (panels B-F). In F, the indicated statistical significance was the same for both stretch and stress. The axial stress-stretch curves shown in panel B and in Figure S1A-C were taken from the same mice and biomechanical testing but at different pressures.

Figure 2. Effect of EGTA on passive arterial mechanics in WT carotid arteries. Right carotid arteries isolated from male WT (C57BL/6J) mice (n=5) were analyzed by pressure myography and then immediately reanalyzed after treatment with 2 mM EGTA. (A) In vitro stretch as determined from axial force-length tests. (B) Axial stretch-stress curve obtained at 100 mm Hg. (C-E) Changes in vessel outer diameter, inner radius and wall thickness, respectively, with pressure. (F) Circumferential stretch-stress curves with data points showing results at 0-140 mm Hg in increments of 10 mm. Statistical significance was determined by 2-tailed t-test (panel A) or 2-way ANOVA (panels B-F). In F, the indicated statistical significance was the same for both stretch and stress. The axial stress-stretch curves shown in panel B and in Figure S1A-C were taken from the same mice and biomechanical testing but at different pressures.
Figure 3. Deletion of SMC FAK. (A) Images of carotid artery cross-sections from FAK<sup>fl/fl</sup>;<i>iCre</i> mice that had been treated with vehicle (ethanol) or tamoxifen and stained with H&E and an antibody to FAK. Scale bars = 100 μm. Dashed lines in panel A approximate the positions of the external and internal elastic laminae, which separate the medial (M) and adventitial (Adv) layers. (B) The immunostaining results in A were quantified and graphed as box plots with Tukey whiskers; n=6-7. Statistical significance was determined by 2-tailed Mann-Whitney tests comparing ethanol to tamoxifen within the media and adventitia.

Figure 4. Deletion of SMC FAK attenuates circumferential arterial stiffness. Freshly isolated right carotid arteries from FAK<sup>fl/fl</sup>;<i>iCre</i> mice were treated with ethanol (EtOH) or tamoxifen (Tamox) and analyzed by pressure myography (n=5 per condition). (A) In vitro stretch as determined from axial force-length tests. (B) Axial stretch-stress curves obtained at 100 mm Hg. (C) Changes in outer diameter with increasing pressure. (D-E) Changes in vessel inner radius and wall thickness, respectively, with pressure. (F) Circumferential stretch-stress curves with data points showing results at 0-140 mm Hg in increments of 10 mm. Statistical significance was determined by 2-tailed t-test (panel A) or 2-way ANOVA (panels B-F). In F, the indicated statistical significance was the same for both stretch and stress. The axial stress-stretch curves shown in panel B and in Figure S1G-I were taken from the same mice and biomechanical testing but at different pressures.

Figure 5. Arterial fibrillar collagens abundance is not affected by deletion of SMC FAK. (A) Images of carotid artery cross-sections from FAK<sup>fl/fl</sup>;<i>iCre</i> mice that had been treated with vehicle (ethanol) or tamoxifen and stained with antibodies to collagen-I, collagen-III, or collagen-V. Scale bars = 100 μm. Dashed lines approximate the positions of the external and internal elastic
laminae, which separate the medial (M) and adventitial (Adv) layers. (B) The immunostaining results in A were quantified and graphed as box plots with Tukey whiskers; n=5-7. Statistically significant differences were not detected when comparing (by 2-tailed Mann-Whitney tests) ethanol versus tamoxifen treatment within each tissue layer.

Figure 6. Similar expression of SMC differentiation markers upon deletion of SMC FAK. (A) Images of carotid artery cross-sections from FAK<sup>fl/fl</sup>;iCre mice that had been treated with vehicle (ethanol) or tamoxifen and immunostained with antibodies to SM-MHC or SMA show `medial (M) localization of the two contractile markers. Scale bars = 100 μm. (B) Immunostaining results in A were quantified and graphed as box plots with Tukey whiskers; n=7. Statistically significant differences were not detected when comparing (by 2-tailed Mann-Whitney tests) ethanol versus tamoxifen treatment within the media and adventitia (Adv).

Figure 7. Deletion of SMC FAK reduces carotid arterial contraction. FAK<sup>fl/fl</sup>-iCre mice were treated with ethanol or tamoxifen. Freshly isolated left carotid arteries were mounted on the pressure myograph and submerged in HBSS. Mounted vessels were preconditioned by stretching them axially to 1.15 times their UVL at 40 mm Hg for 15 min and then 1.3 times their UVL at 60 mm Hg for 15 min. The preconditioned vessels were brought to their IVS and pressurized to 100 mm Hg. Increasing concentrations of KCl (10<sup>-4</sup> M to 10<sup>-1</sup> M in HBSS; 5 ml) were added to the vessel chamber sequentially at 10-min intervals or once the constriction plateau had been reached. Each new KCl solution was added after removing the prior KCl solution, and the extent of contraction to each KCl solution (defined as the change in outer diameter relative to baseline) was measured when the vessels outer diameter had reached its plateau (~5 min after each treatment). The baseline (dashed line) was defined as the contraction seen with 10<sup>-4</sup> M KCl (this concentration approximated the KCl concentration in HBSS) and set to zero. Data were passed
through a Grubb’s test and then analyzed for statistical significance by 1-way ANOVA; n=6 for ethanol- and n=7-8 for tamoxifen-treated arteries.

**Figure 8. Blebbistatin eliminates the effect of SMC FAK deletion on arterial wall thickness and circumferential stiffness.** Each carotid arteries tested in Fig. 4 was immediately treated with blebbistatin and reanalyzed. (A) In vitro stretch as determined from axial force-length tests. (B) Axial stretch-stress curves obtained at 100 mm Hg. (C) Changes in outer diameter with increasing pressure. (D-E) Changes in vessel inner radius and wall thickness, respectively, with pressure. (F) Circumferential stretch-stress curves with data points showing results at 0-140 mm Hg in increments of 10 mm. Statistical significance was determined as in Fig. 4. In F, statistical significance is shown for stretch; differences in stress were not significant. The axial stress-stretch curves shown in panel B and in Figs. S1J-L were taken from the same mice and biomechanical testing but at different pressures.
Figure 1

A. Bar chart showing in vivo stretch for WT pre-blebbi and WT post-blebbi.

B. Graph showing axial stress (kPa) versus axial stretch for WT pre-blebbi and WT post-blebbi.

C. Graph showing outer diameter (µm) versus pressure (mm Hg) for WT pre-blebbi and WT post-blebbi.

D. Graph showing inner radius (µm) versus pressure (mm Hg) for WT pre-blebbi and WT post-blebbi.

E. Graph showing wall thickness (µm) versus pressure (mm Hg) for WT pre-blebbi and WT post-blebbi.

F. Graph showing circumferential stress (kPa) versus circumferential stretch for WT pre-blebbi and WT post-blebbi.
Figure 2

A

In vivo stretch

WT pre-EGTA  WT post-EGTA

Pressure (mm Hg)

WT pre-EGTA
WT post-EGTA

B

Axial stress (kPa)

WT pre-EGTA
WT post-EGTA

Axial Stretch

C

Outer diameter (µ)

WT pre-EGTA
WT post-EGTA

Pressure (mm Hg)

D

Inner radius (µ)

WT pre-EGTA
WT post-EGTA

Pressure (mm Hg)

E

Wall thickness (µ)

WT pre-EGTA
WT post-EGTA

Pressure (mm Hg)

F

Circumferential stress (kPa)

WT pre-EGTA
WT post-EGTA

Circumferential stretch
Figure 3

A

EtOH

Tamox

H&E

Lumen

Lumen

FAK

Lumen

Lumen

B

FAK signal intensity (a.u.)

**

E T E T

Media Adventitia

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Figure 4
Figure 5

A  

Collagen-I  Collagen-III  Collagen-V

EtOH  

M  Adv

Tamox  

M  Adv

B  

Signal intensity (a.u.)

0  10000  20000  30000  40000  50000

E  T  E  T  E  T  E  T

Med  Adv

Collagen-I  Collagen-III  Collagen-V
Figure 6

A

EtOH

Tamox

SM-MHC

SMA

Lumen

Lumen

B

Signal intensity (a.u.)

SM-MHC

SMA

Media
Adv

Media
Adv
Figure 7

Concentration of KCl (M)

Percent contraction

10^{-3} 10^{-2} 10^{-1} 10^{-3} 10^{-2} 10^{-1}

-5 0 5 10 15

Ethanol
Tamoxifen

***

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Figure 8

A

B

C

D

E

F

In vivo stretch

Axial stress (kPa)

Axial stretch

Pressure (mm Hg)

Outer diameter (µm)

Inner radius (µm)

Wall thickness (µm)

Circumferential stretch

Pressure (mm Hg)

Circumferential stress (kPa)

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