Caveolin-1 Facilitates Mechanosensitive Protein Kinase B (Akt) Signaling In Vitro and In Vivo

Daniel G. Sedding, Jennifer Hermse, Ulrike Seay, Oliver Eickelberg, Wolfgang Kummer, Carsten Schwencke, Ruth H. Strasser, Harald Tillmanns, Ruediger C. Braun-Dullaeus

Abstract—Mechanotransduction represents an integral part of vascular homeostasis and contributes to vascular lesion formation. Previously, we demonstrated a mechanosensitive activation of phosphoinositide 3-kinase (PI3-K)/protein kinase B (Akt) resulting in p27Kip1 transcriptional downregulation and cell cycle entry of vascular smooth muscle cells (VSMC). In this study, we further elucidated the signaling from outside-in toward PI3-K/Akt in vitro and in an in vivo model of elevated tensile force. When VSMC were subjected to cyclic stretch (0.5 Hz at 125% resting length), PI3-K, Akt, and Src kinases were found activated. Disrupting caveolar structures with β-cyclodextrin or transfection of VSMC with caveolin-1 antisense oligonucleotides (ODN) prevented PI3-K and Akt activation and cell cycle entry. Furthermore, PI3-K and Akt were resistant to activation when Src kinases were inhibited pharmacologically or by overexpression of a kinase-dead c-Src mutant. αβ integrins were identified to colocalize with PI3-K/caveolin-1 complexes, and blockade of αβ integrins prevented Akt activation. The central role of caveolin-1 in mechanotransduction was further examined in an in vivo model of elevated tensile force. Interposition of wild-type (WT) jugular veins into WT carotid arteries resulted in a rapid Akt activation within the veins that was almost abolished when veins of caveolin-1 knockout (KO) mice were used. Furthermore, late neointima formation within the KO veins was significantly reduced. Our study provides evidence that PI3-K/Akt is critically involved in mechanotransduction of VSMC in vitro and within the vasculature in vivo. Furthermore, caveolin-1 is essential for the integrin-mediated activation of PI3-K/Akt. (Circ Res. 2005;96:635-642.)

Key Words: remodeling ■ muscle, smooth ■ signal transduction ■ stress ■ vasculature

It is currently recognized that the machinery governing the cell cycle regulates multiple cellular functions in the cardiovascular system, thereby maintaining the homeostasis of the vasculature and allowing its adaptation to acute and chronic changes. Besides organizing cellular proliferation, the cell cycle is involved in migration, apoptosis, and hypertrophy.1

One of the major constituents of the blood vessel wall responsible for the maintenance of vessel structures and functions are vascular smooth muscle cells (VSMCs). In the vasculature, VSMCs are constantly exposed to alternating mechanical forces. Under normal tensile stress, VSMCs are relatively insensitive to mitogens. During altered mechanical stress (eg, high blood pressure), however, VSMCs upregulate protein synthesis in response to growth factors, dedifferentiate, and increase their proliferative rate, resulting in medial hypertrophy and intimal hyperplasia.2 Whereas the commonly accepted “response to injury” hypothesis suggests that growth factors are locally released, thereby initiating cell cycle entry and progression of vascular cells, the signaling pathways arising solely from mechanical force have just partially come to light.1 Recently, we demonstrated that the earliest cell cycle events can occur in a mechanosensitive fashion independently of newly released or synthesized growth factors but that they are dependent on an intact integrin signaling.4

Besides mediating cell adhesion, integrins transmit extracellular stimuli into intracellular signaling events.3 Thus, integrins mediate VSMC dedifferentiation, migration, proliferation, and apoptosis.6 Signaling through integrins requires physical interaction with other membrane proteins and subsequent association with signal transduction proteins of the cytoplasm. Caveolin-1 functions as a membrane adaptor. For example, caveolin-1 links the integrin α-subunit to the c-Src kinase pathway and subsequently to the mitogen-activated protein kinase pathway (MAPK) to promote cell cycle progression.7 Another pathway critical for VSMC proliferation consists of the phosphoinositide 3-kinase(Pi3-K)/protein ki-
nase B (Akt) signal transduction pathway. Although the MAPK pathway had previously been shown to be responsive to mechanical force, we were recently able to demonstrate that cyclic stretch resulted in a rapid PI3-K/Akt activation as well, thereby inducing forkhead transcription factor–dependent downregulation of the cell cycle inhibitor p27Kip1. This chain of events facilitated mechanosensitive cell cycle entry and proliferation of VSMCs in contrast to the posttranscriptional downregulation of p27Kip1 protein in VSMCs stimulated with serum mitogens. In this way, p27Kip1 may serve as a differential sensor for growth factor– and mechanically induced cell cycle entry in VSMCs. In the present study, we further elucidated the integrin/adaptor protein interactions resulting in growth factor–independent PI3-K/Akt activation and subsequent cell cycle entry. We present evidence that the structural protein caveolin-1 is essential for integrin-mediated PI3-K/Akt activation during cyclic stretch of VSMCs in vitro and in an in vivo model of elevated tensile force. In our in vitro model, mechanical force induces the formation of functional signaling complexes composed of αβ integrins, caveolin-1, PI3-K/Akt, and the nonreceptor tyrosine kinase c-Src, resulting in PI3-K/Akt activation and thereby facilitating a mechanosensitive proliferative response of VSMCs.

Materials and Methods

Cell Culture, Stretch Apparatus, and Experimental Conditions
Primary cultures of VSMCs were initiated by enzymatic dissociation from the aorta of 7- to 8-week-old male Sprague-Dawley rats (Charles River Breeding Laboratories, Kingston, NY). The cells were seeded (~10,000 cells/cm²) onto 6-well fibronectin-coated Flexi plates (Flexcell). Studies were conducted on VSMCs (passages 7 to 12) after achieving confluence in 10% FCS/DMEM/F12, FlexI plates (Flexcell). Studies were conducted on VSMCs (passages 7 to 12) after achieving confluence in 10% FCS/DMEM/F12, following by serum withdrawal for 2 days to achieve quiescence. On the day of the experiment, fresh serum-free medium was substituted and uniaxial cyclic stretch was applied with a flexercell apparatus.

Immunocytochemistry
VSMCs were grown on fibronectin-coated flexercell wells. Quiescent or stretched (30 minutes) cells were fixed for 10 minutes in ice-cold acetone. Cells were covered for 20 minutes with 10% normal goat serum, followed by incubation with rabbit anti-caveolin-1 (1:100) and mouse anti p38 (p85, 1:50) for 1 hour in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. After two washing steps (10 minutes in PBS), cells were incubated with secondary antibodies for 2 days to achieve quiescence. On the day of the experiment, fresh serum-free medium was substituted and uniaxial cyclic stretch was applied with a flexercell apparatus (125% resting length, 0.5 Hz) in a tissue culture incubator.

Immunoblot Analysis
VSMCs were grown on fibronectin-coated flexercell wells. Quiescent or stretched (30 minutes) cells were fixed for 10 minutes in ice-cold acetone. Cells were covered for 20 minutes with 10% normal goat serum, followed by incubation with rabbit anti-caveolin-1 (1:100) and mouse anti p38 (p85, 1:50) for 1 hour in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. After two washing steps (10 minutes in PBS), cells were incubated with secondary antibodies for 2 days to achieve quiescence. On the day of the experiment, fresh serum-free medium was substituted and uniaxial cyclic stretch was applied with a flexercell apparatus. After washing, cells were mounted in Vectashield mounting medium H-1000 containing DAPI and evaluated using an epifluorescence microscope (DMRB, Leica). Negative controls were performed using only the secondary antibody.

Preparation of Cellular Lysates and Immunoblot Analysis
Specific protein content in cell lysates was analyzed by immunoblot as previously described.11 Briefly, cell lysis buffer contained 50 mmol/L HEPES pH 7.4, 100 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L glycerophosphate, 2 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L vanadate, 0.5% octylglucopyranoside, PMSF 100 μg/mL, aprotonin 30 μL/mL, leupeptin 1 μg/mL, and okadaic acid 10 μmol/L. Lysate proteins (20 μg) were run on a polyacrylamide gel and blotted onto nitrocellulose (Hybond-ECL, Amersham). After blocking, blots were incubated with primary antibody for 1 hour at room temperature. Specific proteins were detected by enhanced chemiluminescence (ECL+, Amersham) after labeling with horse-radish peroxidase-labeled secondary antibody (1:2000 for 1 hour) according to the manufacturer’s instructions.

Flow Cytometry
Cells were harvested by trypsinization, fixed overnight with 75% methanol, washed, and incubated with 100 μg/mL RNase (Oncogene Research Products) and 10 μg/mL propidium iodide in PBS for 1 hour at 37°C. Samples were analyzed for DNA content using a high-speed cell sorter (EPics Altra, Beckman Coulter). Data were computer-analyzed with commercially available software (Multicycle, Phoenix Flow Systems).

Mice and Vein Graft Procedure
Caveolin-1–/- mice were provided by M. Drab, Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany. All animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals (Giessen University). The vein graft procedure has been described previously.12 Briefly, mice were anesthetized with ketamine (50 mg/kg body weight, IP). The jugular vein was harvested from the donor. In the recipient, the left common carotid artery was mobilized from proximal to the distal bifurcation, cut in the middle, and a cuff (0.63 mm outside diameter and 0.5 mm inside diameter, Portex LTD) placed at each end. At each end, the artery was turned inside out over the cuff and ligated. The vein segment was grafted between the two ends of the carotid artery by sleeving the ends of the vein over the artery-cuff and ligating them together with an 8-0 suture.

For Western blot experiments, vein grafts were perfused with PBS, excised immediately, and snap-frozen in liquid nitrogen until further use.

Statistical Analysis
Data are given as mean±SEM. Statistical analysis was performed by ANOVA. Post hoc analysis was performed by the method of Bonferroni. All experiments, including the immunoblots, were independently repeated at least three times.

For detailed description of reagents, transfection procedures, magnetic activated cell sorting (MACS), phosphoinositide 3-kinase assay, Src family kinase assay, and histological evaluation of tissue sections please refer to the online data supplement available at http://circres.ahajournals.org.

Results
Mechanical Strain Induces Colocalization of PI3-K With Caveolin-1 and Their Clustering at Focal Adhesion Sites
Previously, we had demonstrated that stretch-induced cell cycle entry and progression can be prevented by pharmacological inhibition of PI3-K (LY294002 and wortmannin) or overexpression of a constitutive negative Akt, indicating a requirement for PI3-K/Akt in mechanically induced proliferation of VSMCs.4 In the present examination of cellular distribution, it was found that in quiescent cells, PI3-K had a homogeneous cytosolic distribution and that caveolin-1 was distributed at the cell surface in a diffuse, punctuate manner (Figure 1A and 1B). After 15 minutes of cyclic stretch, PI3-K and caveolin-1 accumulated in clusters at the cell membrane. Double-staining suggested their colocalization (Figure 1A and 1B). The PI3-K/caveolin-1 complexes seemed to be located at focal adhesion sites as implicated by their colocalization with vinculin (shown for caveolin-1; Figure 1A and 1B).
Mechanosensitive Activation of PI3-K/Akt Requires Intact Caveolar Structures and Caveolin-1

We examined whether caveolar structures are required for the mechanosensitive activation of PI3-K/Akt. Addition of β-cyclodextrin, which depletes cholesterol from caveolae and thereby disrupts caveolar structures, was able to prevent stretch-induced assembly of PI3-K with Akt and caveolin-1 (Figure 2A). Furthermore, it prevented activation of PI3-K and phosphorylation of Akt (Figure 2B). Because caveolin-1 essentially contributes to caveolae formation and serves as an adaptor molecule for multiple cell membrane molecules, involvement of caveolin-1 in stretch-induced PI3-K activation and Akt phosphorylation was examined. Incubation of cells with caveolin-1 antisense oligonucleotides (cav-1 AS ODN) but not control (reverse) ODN resulted in an almost complete abolition of caveolin-1 expression within 24 hours (Figure 2C). Incubation with cav-1 AS ODN 24 hours before stretch was able to completely prevent phosphorylation and, thereby, activation of Akt (Figure 2D).

Activation of PI3-K/Akt is essential for stretch-induced cell-cycle entry and subsequent proliferation of VSMCs.4 Consistent with experiments described earlier demonstrating a caveolin-1–dependent activation of PI3-K/Akt during cyclic stretch, caveolin-1 AS ODN, but not control ODN, almost completely prevented stretch-induced proliferation (Figure 3A). In contrast, caveolin-1 AS ODN did not significantly alter the proliferative response of VSMCs exposed to serum stimulation (10% FCS; Figure 3B). Our data indicate that caveolin-1 mediates mechanosensitive cell cycle entry and progression of VSMCs via PI3-K/Akt.

To further substantiate the results seen by transient AS ODN-mediated caveolin-1 disruption, we isolated VSMCs from aortas of caveolin-1−/− mice (cav-1−/−). As demonstrated in rat VSMCs, exposure of mouse wild-type (WT) VSMCs to cyclic stretch for 15 minutes induced a profound activation of Akt as well as p42/44 (Erk) mitogen-activated protein kinase (MAPK) (Figure 3C). This activation was comparable to the activation seen in cells exposed to 10% FCS in the absence of cyclic stretch. Akt activation was...
almost abolished when VSMCs of cav-1/H11002/H11002 mice were used (Figure 3C). Although a moderate Akt activation was detected after addition of 10% FCS to cav-1/H11002/H11002 VSMCs, this activation was markedly impaired, suggesting that caveolin-1 also facilitates growth factor–induced Akt activation. Similarly, Erk activation was impaired in cav-1/H11002/H11002 cells exposed to cyclic stretch, suggesting that caveolin-1 is also essential for mechanosensitive Erk activation. However, Erk was still sensitive to serum stimulation in these cells. Twenty four hours of cyclic stretch did not trigger proliferation of cav-1/−/− cells but of WT cells (15±1.7 versus 28±2.4 of cells in S/G2-phase, respectively; n=4, *P<0.01). C, Quiescent or stretched VSMCs isolated from wild-type C57/BL6J mice or caveolin-1/−/− mice were evaluated for Akt or ERK phosphorylation by immunoblotting. Caveolin-1 expression was observed in wild-type cells only. PanAkt expression served as a loading control. D, Number of proliferating cells (cells in G2/S phase) was determined by FACS analysis (black bars, wild-type; gray bars, cav-1/−/−; n=4; *P<0.01).

Cyclic Stretch Causes Activation of Src Family Kinases, Their Association With Caveolin-1 and PI3-K (p85), and Activation of PI3-K/Akt
Src nonreceptor protein tyrosine family kinases have been demonstrated to associate with caveolin-1.13 Src family kinases (SFKs) have also been shown to be involved in Akt activation.14 We examined whether the SFK c-Src, Fyn, or c-Yes associate with caveolin-1 and PI3-K (p85) under conditions of cyclic stretch. Lysates of rat VSMCs stretched for 5 minutes were immunoprecipitated with anti-c-Src, anti-Fyn, or anti-c-Yes, and an immunoblot was performed for caveolin-1. Cyclic stretch rapidly stimulated caveolin-1/Fyn, caveolin-1/c-Src, and caveolin-1/c-Yes association in VSMCs (Figure 4A). Furthermore, cyclic stretch resulted in activation of Fyn, c-Src, and c-Yes as examined by their autophosphorylation (Figure 4B). SFK activation was rapid and transient, as peak activity levels were already reached after 30 minutes of cyclic stretch (data not shown). As expected, stretch-induced activation of SFKs was prevented by the specific inhibitor, PP1. In addition, cyclic stretch resulted in a significant (4.5-fold) increase of c-Src tyrosine kinase activity (Figure 4C, n=3, *P<0.01).

Figure 4. Cyclic stretch causes association of Src family kinase (SFK) with caveolin-1 and results in SFK activation. A, VSMCs were exposed to cyclic stretch or 10% FCS for 24 hours. Cells were pretreated with caveolin-1 AS ODN (AS ODN) or reverse ODN (rev ODN) 24 hours before stretch. After fixation and propidium iodide staining, cell cycle distribution was determined by FACS analysis. Number of proliferating cells (cells in G2/S phase) was evaluated (n=4, *P<0.01). C, Quiescent or stretched VSMCs isolated from wild-type C57/BL6J mice or caveolin-1/H11002/H11002 mice were evaluated for Akt or ERK phosphorylation by immunoblotting. Caveolin-1 expression was observed in wild-type cells only. PanAkt expression served as a loading control. D, Number of proliferating cells (cells in G2/S phase) was determined by FACS analysis (black bars, wild-type; gray bars, cav-1/−/−; n=4; *P<0.01).
cyclic stretch. Infected cells were then plated and exposed to 15 minutes of 24 hours before positive selection by MACS. Positively transduced cells derived from cav-1-/- mice were exposed to arterial pressure for 15 minutes, activation (phosphorylation) of Akt and Erk was determined by immunoblotting. Lysates of cells stretched for 10 minutes were immunoprecipitated with anti–PI3-K (p85), and immunoblots were performed for αv, β3 integrins. Both integrin types were found to be associated with PI3-K (p85), indicating that αv or β3 integrins play a role in mechanically induced PI3-K/Akt activation (Figure 6A). To further examine αvβ3 integrin involvement in PI3-K/Akt activation, we preincubated cells with a specific αvβ3-integrin inhibitor (XJ735). Preincubation of cells with XJ735 but not with a control peptide prevented stretch-induced Akt activation (Figure 6B), indicating that stretch-induced PI3-K activation is mediated to a large extent by αvβ3 integrins.

Mechanosensitive Akt Activation and Neointima Formation Is Impaired in Caveolin-1-/- Mice

To validate the role of caveolin-1 in mechanical strain–induced signal transduction and proliferation in vivo, jugular vein segments from cav-1-/- mice were exposed to arterial pressure by transplantation into the mouse common carotid artery of C57BL6j WT recipients. After exposure of the vein grafts to arterial pressure for 15 minutes, activation (phosphorylation) of Akt and Erk was determined by immunoblotting. The results indicate that Akt activation was prevented in the veins of cav-1-/- mice (Figure 7A). Similarly, Erk activation was impaired in the veins of cav-1-/- mice compared with the veins of WT controls (Figure 7A).

To evaluate if impaired force-induced Akt (and Erk) activation of cav-1-/- VSMCs in vitro and in vivo finds a correlate in an altered neointima formation, vein segments were interposed into the arterial system for 6 weeks. Whereas veins of WT mice developed severe neointimal lesions, neointima formation was significantly reduced in veins derived from cav-1-/- mice (0.12±0.03 versus 0.19±0.03 mm², respectively; n=8, P<0.01; Figure 7B and 7C).

Discussion

Mechanotransduction plays a critical role in vascular homeostasis. Whereas physiologically moderate cyclic stretch seems essential for maintaining vessel wall structure and for...
inhibition of growth factor–stimulated proliferation of VSMCs, enhanced tensile force, more likely resembling pathological conditions such as those occurring in severe hypertension, venous bypass grafts, or during balloon angioplasty, has been reported to induce proliferation of VSMCs. In contrast to growth factor–induced cell cycle entry and proliferation, the mechanosensitive signaling events from the cell surface toward the cell cycle machinery are still poorly understood. Recently, we were able to demonstrate that mechanical force activates PI3-K/Akt signaling independent of growth factors. Akt in turn inactivates forkhead transcription factors, which are involved in p27Kip1 gene transactivation. Subsequent downregulation of p27Kip1 resulted in cell cycle entry and progression of VSMCs.

In the present study, we were able to further characterize the components and mechanisms of VSMCs mechanosensing that initiate mechanically induced proliferation of VSMCs. In contrast to growth factor–induced cell cycle entry and proliferation, the mechanosensitive signaling events from the cell surface toward the cell cycle machinery are still poorly understood. Recently, we were able to demonstrate that mechanical force activates PI3-K/Akt signaling independent of growth factors. Akt in turn inactivates forkhead transcription factors, which are involved in p27Kip1 gene transactivation. Subsequent downregulation of p27Kip1 resulted in cell cycle entry and progression of VSMCs.

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we demonstrate the relevance of our findings in an in vivo model of enhanced tensile force. Interposing a vein of a cav-1-/- mouse into the carotid artery of a WT mouse allowed us to examine the effect of force on the cav-1-/- vasculature independent of systemic limitations inherent in caveolin-1 gene-disrupted mice. In cav-1-/- veins, Akt activation was completely abolished. Nevertheless, veins of cav-1-/- mice still developed a neointima, although to a significantly lesser extent. Previous studies and our in vitro data may explain the in vivo results: whereas Akt was unresponsive to both serum and mechanical stimulation when caveolin-1 was deleted, exposure of these cells to serum mitogens still resulted in Erk activation. On the other hand, stretch-induced proliferation was completely prevented when VSMCs of cav-1-/- mice were used, whereas growth factor treatment still resulted in their cell cycle entry and progres-
sion. This points toward Akt’s predominant role for mechanically triggered proliferation. Indeed, pharmacological inhibition of Erk during stretch did not impair the proliferative response of VSMCs in vitro. Furthermore, our study also points toward a predominant role of caveolin-1 for mechanosensitive cell cycle entry and progression. Growth factor–induced proliferation, however, was not impaired when caveolin-1 was disrupted, a finding previously described.

In vivo, the net effect of growth factors and mechanically triggered stimuli determine the amount of neointima formation. This may explain why late neointima formation was significantly reduced but not abolished in our model. Moreover, it may explain why another study that used instead an inflammatory model of neointima formation reported an increase of lesion size in cav-1−/− mice. Obviously, more factors have to be considered. In cav-1−/− mice, endothelial nitric oxide synthase (eNOS) is activated, eNOS gene transfer has been shown to prevent neointima formation in denuded rat carotid arteries. Therefore, augmented endothelial nitric oxide synthesis may be additionally responsible for reduced neointima formation in our in vivo model as well as reduced plaque formation in ApoE−/− mice interbred with cav-1−/−.

As mentioned earlier, other studies have demonstrated that signaling components within caveolae may be held inactive until their activation and release by appropriate external stimuli. The p42/44 (Erk) MAP kinase cascade has been shown to be predominantly negatively regulated by caveolin. Moreover, mice lacking caveolin-1 have defects in nitric oxide and calcium signaling, and their lungs display severe abnormalities caused by uncontrolled cell proliferation and fibrosis, pointing toward the role of caveolin in inhibiting signaling pathways that regulate cellular proliferation in lung tissue. It is not yet clear, however, why hyperproliferative abnormalities were not found in other tissues that are normally rich in caveolin-1. This may be explained by studies demonstrating that caveolae can also stimulate signaling activity and proliferation. The association of caveolin-1 with the integrin α-subunit and the tyrosine kinase Fyn, for example, leads to activation of the Ras-Erk pathway and promotes cell cycle progression. The PDGF and the insulin receptors also seem to initiate their signal transduction from caveolae. Our data support the hypothesis that in the vasculature, which is constantly exposed to alternating mechanical force and different growth factors, a dual role of caveolin-1 may contribute to a sensitive balance of anti- and pro-proliferative effects that allow the vessel to adapt to acute or chronic changes. Interestingly, a dual role of caveolin-1 toward eNOS has recently been demonstrated: although caveolin-1 repressed basal eNOS activity, it was crucial for agonist- (vascular endothelial growth factor–) induced eNOS activation.

In this study, we also demonstrate that cholesterol depletion of the plasma membrane by β-cycloexodextrin prevents PI3-K/Akt activation. Caveolae form from cholesterol- and sphingolipid-rich rafts in the membrane in a process that requires caveolin-1. Furthermore, the function of caveolae is dependent on a sufficient level of cholesterol in the plasma membrane, and caveolin-1 is involved in maintaining caveolar cholesterol levels. Consistent with our results, reduction of plasma membrane cholesterol levels with cholesterol-binding agents has been shown to inhibit specific agonist-stimulated signaling pathways, eg, insulin-stimulated insulin receptor substrate-1 phosphorylation or shear stress, and growth factor–induced and endothelin–induced activation of the extracellular signal-regulated kinase, Erk. Although our data suggest that caveolin-1 acts directly through protein-protein interactions, it may also operate indirectly by maintaining caveolar structure and integrity necessary for mechanosensitive proliferation through integrin/c-Src/PI3-K/Akt interaction.

Immunocytochemical staining of VSMCs indicates a force-dependent recruitment of PI3-K and caveolin-1 to focal adhesion sites, suggesting that PI3-K plays a role in integrin-mediated cellular response to mechanical stimuli. It has been shown that integrins are, indeed, sequestered into caveolar microdomains of the plasma membrane, implying a requirement of caveolar microdomains for integrating integrin-mediated mechanotransduction toward an intracellular signal. In VSMCs, the proliferative response to strain was abrogated by antibodies to both α1 and β1 integrins, but not β2 integrins. In accordance with these findings, we find a dependency of tensile stress-mediated PI3-K/Akt activation on α1β1 integrins. Furthermore, Src family kinases are required for the force-dependent formation of focal adhesion complexes and strengthening of α1β1 integrin-cytoskeleton connections. In addition, an involvement of Src family kinases in PI3-K and in Akt activation has been reported before. In this study, we demonstrate that a pharmacological inhibitor of the Src kinase family tyrosine kinases (PP1) prevents not only Akt activation and proliferation, but also PI3-K activation, indicating that mechanosensitive c-Src kinase signaling lies upstream of PI3-K. Our findings support the concept that c-Src is an additional, essential component for mechanosensitive PI3-K activation.

Mechanosensing of VSMCs via α1β2 integrins/caveolin-1/c-Src kinase–dependent activation of PI3-K/Akt signaling and subsequent cellular proliferation may play an important role in physiological vascular remodeling processes and the pathophysiology of vascular proliferative diseases. Our data further add to the understanding of mechanisms involved in vascular homeostasis and the pathophysiology of proliferative disease processes and may have novel implications for the future design of therapeutic interventions.

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Materials and Methods

Reagents

The following antibodies were used: rabbit anti-phospho-Akt (ser473, pAkt) (New England Biolabs, Frankfurt, Germany), sheep anti-panAkt1 (Upstate Biotech, Waltham, Massachusetts), rabbit anti-caveolin-1, rabbit anti-c-Src, mouse anti-Fyn, rabbit anti-c-Yes, rabbit anti-integrin αV, rabbit anti-integrin β3, mouse anti-Cdk4, rabbit anti-vinculin (Santa Cruz Biotechnology, Santa Cruz, California, USA), mouse anti-PI3-K(p85) (BD Biosciences, Erembodegem, Belgium). Secondary antibodies used were goat anti-rabbit IgG and goat anti-mouse IgG, both linked to horseradish peroxidase (Santa Cruz Biotechnology Inc. Santa Cruz, California). Cyclodextrin and the Src kinase inhibitor PP1 were obtained from Biomol, Hamburg, Germany. Specific integrin αVβ3 inhibitor XJ735 and control peptide (GRGESP) were from Bachem, Weil am Rhein, Germany. 4’,6-Diamidino-2-phenylindole, 2HCl (DAPI) was purchased from Calbiochem, San Diego, USA. Plasmids: kinase-inactive pUSEamp-c-Src(K297R) was obtained from Upstate, Lake Placid, New York, and pIRES2-EGFP was purchased from Clontech, Palo Alto, CA, USA.

Transfection Procedures and Magnetic-Activated Cell Sorting (MACS)

For caveolin-1 antisense oligonucleotide (ODN) experiments, phosphorothioate ODN were modified by the addition of a propinyl group to the pyrimidine base cytidine, which is thought to enhance base stacking for enhanced sense antisense interaction. The antisense ODN sequence used in the experiments was 5’-ACCCCCAGACATGCTGGC-3’ (complementary to bases 23-40 of rat caveolin-1; Z46614); the reverse ODN sequence was 5’-CGGTCGTACAGACCCCCA-3’. For the lipofection procedure, 20 nmol/l ODN were mixed with Fugene (Roche, Mannheim, Germany) in serum-free medium (Opti-MEM, GIBCO BRL,
Karlsruhe, Germany) and incubated for 10 min. VSMC were rinsed once with PBS and the ODN-Fugene solution was added to the cells in a serum-free medium. Cells were then incubated for 24 h before the experiment was performed as indicated. Subsequently, the cells were harvested by trypsinization for FACS analysis or Western blotting. FITC-labeled ODN showed nuclear uptake in up to 90% of cells using this technique (data not shown).

For MACS, cells were co-transfected with equimolar amounts of pMACS.K^k-II and the expression plasmid in a 1:3 ratio as indicated. Transfection was carried out using Fugene (Roche, Mannheim, Germany). For a 35-mm dish, 6 µl liposomes were added to 100 µl Opti-MEM (GIBCO BRL, Karlsruhe, Germany) and mixed with 2 µg vector DNA before adding the mixture to the cells in a serum-free medium. After 4 h, medium was withdrawn and fresh medium containing 10% FCS was added. Transfection efficiency was 20-30%. After 24 h, transfected cells were trypsinized and magnetically labeled using MACSelect K^k-II MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Transfected (K^k-II positive) cells were then separated on MS^+/RS^+ separation columns (Miltenyi Biotec) and subsequently replated on flexercell-plates. For analysis, cells were lysed in the appropriate buffer. Positively selected cells were >85% positive for K^k-II expression.

**Phosphoinositide 3-Kinase Assay**

Cells were washed and lysed as described above. The supernatant was collected and approximately 500 µg of soluble proteins were subjected to immunoprecipitation with the indicated antibodies for 2 h at 4 °C. The immunocomplexes were harvested by magnetic separation using protein G Dynabeads® (Dynal Biotech, Hamburg, Germany), washed twice in lysis buffer, once in kinase buffer (20 mmol/l Tris pH 7.4, 100 mmol/l NaCl, 0.5 mmol/l EDTA), and incubated with 15 µg of phosphatidylinositol (Sigma). The assay was started by adding 5 µCi of [α-^{32}P]ATP, 20 µmol/l ATP, and 20 mmol/l MgCl_2 for 20 min. The reaction was terminated after 20 min by addition of chloroform-methanol-11.6 mol/l HCl (100:200:2),
after which the lipids were extracted by chloroform:methanol (1:1) and fractioned by thin-
layer chromatography in chloroform-methanol-water-ammoniumhydroxide (43:30:7:5). The
thin-layer chromatography plate was then air-dried and subjected to autoradiography.

**Src Family Kinase (SFK) Kinase Assay**

Cells were lysed as described above. Lysate samples containing 250 µg of protein were pre-
cleared with normal rabbit immunoglobulin G (IgG) bound to protein G Dynabeads® before
incubation with 1 µg of anti-Fyn, anti-c-Src, or anti-Yes antibody for 2 h. Magnetic separation
was achieved using protein G Dynabeads®. Immune complexes were washed 4 times with
lysis buffer, and twice with kinase buffer (50 mmol/l HEPES [pH 7.4], 10 mmol/l MnCl₂, 1
mmol/l dithiothreitol), re-suspended in kinase buffer containing 10 μCi of [α-³²P]ATP and
25 μmol/l ATP, and incubated for 15 min at 30 °C. The reaction was terminated by addition
of Laemmli buffer and subsequent boiling. After electrophoresis, gels were air-dried and
subjected to autoradiography.

For relative quantification of c-Src tyrosine kinase activity, cells were lysed in RIPA buffer
and cell lysates were immunoprecipitated with an anti-c-Src antibody as described above.
Activity of c-Src kinase was determined by a non-radioactive tyrosine kinase assay kit
according to the manufacturer’s protocol (Chemicon, Harrow, UK). Relative changes in c-Src
activity (OD 450) were displayed as means ± s.e.m.

**Histology and Morphometry**

Mice were anesthetized, perfused with 0.9% NaCl solution via cardiac puncture into the left
ventricle for 2 min and subsequently perfusion-fixed with 4% phosphate-buffered
formaldehyde (pH 7.2) for 5 min. Interposed vessels were harvested by cutting the grafted
segments from the native vessels at the cuff end. Vessel samples were fixed with 4%
phosphate-buffered formaldehyde at 4 °C for 24 h. The grafts were embedded in tissue-tec (Miles Laboratories, Naperville, IL) and processed by routine histology. Three sections (4 µm) per graft were stained with hematoxylin and eosin (HE) for histological evaluation and quantification of neointimal area with KS300 software (Carl Zeiss, Hallbergmoos, Germany).
Online Data Supplements:

Materials and Methods

Reagents

The following antibodies were used: rabbit anti-phospho-Akt (ser473, pAkt) (New England Biolabs, Frankfurt, Germany), sheep anti-panAkt1 (Upstate Biotech, Waltham, Massachusetts), rabbit anti-caveolin-1, rabbit anti-c-Src, mouse anti-Fyn, rabbit anti-c-Yes, rabbit anti-integrin αV, rabbit anti-integrin β3, mouse anti-Cdk4, rabbit anti-vinculin (Santa Cruz Biotechnology, Santa Cruz, California, USA), mouse anti-PI3-K(p85) (BD Biosciences, Erembodegem, Belgium). Secondary antibodies used were goat anti-rabbit IgG and goat anti-mouse IgG, both linked to horseradish peroxidase (Santa Cruz Biotechnology Inc. Santa Cruz, California). Cyclodextrin and the Src kinase inhibitor PP1 were obtained from Biomol, Hamburg, Germany. Specific integrin αVβ3 inhibitor XJ735 and control peptide (GRGESP) were from Bachem, Weil am Rhein, Germany. 4´,6-Diamidino-2-phenylindole, 2HCl (DAPI) was purchased from Calbiochem, San Diego, USA. Plasmids: kinase-inactive pUSEamp-c-Src(K297R) was obtained from Upstate, Lake Placid, New York, and pIRES2-EGFP was purchased from Clontech, Palo Alto, CA, USA.

Transfection Procedures and Magnetic-Activated Cell Sorting (MACS)

For caveolin-1 antisense oligonucleotide (ODN) experiments, phosphorothioate ODN were modified by the addition of a propinyl group to the pyrimidine base cytidine, which is thought to enhance base stacking for enhanced sense antisense interaction. The antisense ODN sequence used in the experiments was 5´-ACCCCCAGACATGCTGGC-3´ (complementary to bases 23-40 of rat caveolin-1; Z46614); the reverse ODN sequence was 5´-CGGTCGTACAGACCCCCA-3´. For the lipofection procedure, 20 nmol/l ODN were mixed with Fugene (Roche, Mannheim, Germany) in serum-free medium (Opti-MEM, GIBCO BRL,
Karlsruhe, Germany) and incubated for 10 min. VSMC were rinsed once with PBS and the ODN-Fugene solution was added to the cells in a serum-free medium. Cells were then incubated for 24 h before the experiment was performed as indicated. Subsequently, the cells were harvested by trypsinization for FACS analysis or Western blotting. FITC-labeled ODN showed nuclear uptake in up to 90% of cells using this technique (data not shown).

For MACS, cells were co-transfected with equimolar amounts of pMACS.K\(^k\)-II and the expression plasmid in a 1:3 ratio as indicated. Transfection was carried out using Fugene (Roche, Mannheim, Germany). For a 35-mm dish, 6 µl liposomes were added to 100 µl Opti-MEM (GIBCO BRL, Karlsruhe, Germany) and mixed with 2 µg vector DNA before adding the mixture to the cells in a serum-free medium. After 4 h, medium was withdrawn and fresh medium containing 10% FCS was added. Transfection efficiency was 20-30%. After 24 h, transfected cells were trypsinized and magnetically labeled using MACSelect K\(^k\)-II MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Transfected (K\(^k\)-II positive) cells were then separated on MS\(^+/RS^+\) separation columns (Miltenyi Biotec) and subsequently replated on flexercell-plates. For analysis, cells were lysed in the appropriate buffer. Positively selected cells were >85% positive for K\(^k\)-II expression.

**Phosphoinositide 3-Kinase Assay**

Cells were washed and lysed as described above. The supernatant was collected and approximately 500 µg of soluble proteins were subjected to immunoprecipitation with the indicated antibodies for 2 h at 4 °C. The immunocomplexes were harvested by magnetic separation using protein G Dynabeads® (Dynal Biotech, Hamburg, Germany), washed twice in lysis buffer, once in kinase buffer (20 mmol/l Tris pH 7.4, 100 mmol/l NaCl, 0.5 mmol/l EDTA), and incubated with 15 µg of phosphatidylinositol (Sigma). The assay was started by adding 5 µCi of [\(\alpha\)-\(^{32}\)P]ATP, 20 µmol/l ATP, and 20 mmol/l MgCl\(_2\) for 20 min. The reaction was terminated after 20 min by addition of chloroform-methanol-11.6 mol/l HCl (100:200:2),
after which the lipids were extracted by chloroform:methanol (1:1) and fractioned by thin-layer chromatography in chloroform-methanol-water-ammoniumhydroxide (43:30:7:5). The thin-layer chromatography plate was then air-dried and subjected to autoradiography.

**Src Family Kinase (SFK) Kinase Assay**

Cells were lysed as described above. Lysate samples containing 250 µg of protein were precleared with normal rabbit immunoglobulin G (IgG) bound to protein G Dynabeads® before incubation with 1 µg of anti-Fyn, anti-c-Src, or anti-Yes antibody for 2 h. Magnetic separation was achieved using protein G Dynabeads®. Immune complexes were washed 4 times with lysis buffer, and twice with kinase buffer (50 mmol/l HEPES [pH 7.4], 10 mmol/l MnCl₂, 1 mmol/l dithiothreitol), re-suspended in kinase buffer containing 10 µCi of [α-³²P]ATP and 25 µmol/l ATP, and incubated for 15 min at 30 °C. The reaction was terminated by addition of Laemmli buffer and subsequent boiling. After electrophoresis, gels were air-dried and subjected to autoradiography.

For relative quantification of c-Src tyrosine kinase activity, cells were lysed in RIPA buffer and cell lysates were immunoprecipitated with an anti-c-Src antibody as described above. Activity of c-Src kinase was determined by a non-radioactive tyrosine kinase assay kit according to the manufacturer’s protocol (Chemicon, Harrow, UK). Relative changes in c-Src activity (OD 450) were displayed as means ± s.e.m.

**Histology and Morphometry**

Mice were anesthetized, perfused with 0.9% NaCl solution via cardiac puncture into the left ventricle for 2 min and subsequently perfusion-fixed with 4% phosphate-buffered formaldehyde (pH 7.2) for 5 min. Interposed vessels were harvested by cutting the grafted segments from the native vessels at the cuff end. Vessel samples were fixed with 4%
phosphate-buffered formaldehyde at 4 °C for 24 h. The grafts were embedded in tissue-tec (Miles Laboratories, Naperville, IL) and processed by routine histology. Three sections (4 μm) per graft were stained with hematoxylin and eosin (HE) for histological evaluation and quantification of neointimal area with KS300 software (Carl Zeiss, Hallbergmoos, Germany).