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

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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 \( p27^{kip1} \) 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 \( \beta \)-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. \( \alpha_{\beta} \) integrins were identified to colocalize with PI3-K/caveolin-1 complexes, and blockade of \( \alpha_{\beta} \) 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.

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, dedifferentiation, and increase their proliferative rate, resulting in medial hypertrophy and intimal hyperplasia. 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. 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.

Besides mediating cell adhesion, integrins transmit extracellular stimuli into intracellular signaling events. Thus, integrins mediate VSMC dedifferentiation, migration, proliferation, and apoptosis. 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 \( \alpha_{\beta} \)-subunit to the c-Src kinase pathway and subsequently to the mitogen-activated protein kinase pathway (MAPK) to promote cell cycle progression. Another pathway critical for VSMC proliferation consists of the phosphoinositide 3-kinase (PI3-K)/protein kinase B (Akt) signaling pathway.
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 p27\(^{kip1}\). This chain of events facilitated mechanosensitive cell cycle entry and proliferation of VSMCs in contrast to the posttranscriptional downregulation of p27\(^{kip1}\) protein in VSMCs stimulated with serum mitogens. In this way, p27\(^{kip1}\) 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 \(\alpha\beta\) 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 (\(\sim 10,000\) cells/cm\(^2\)) 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, 7 to 12) after achieving confluence in 10% FCS/DMEM/F12, followed by serum withdrawal for 2 days to achieve quiescence. On the day of the experiment, fresh serum-free medium was substituted.

**Immunocytochemistry**

VSMCs were grown on fibronectin-coated flexcell 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 PI3-K (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 40 minutes: donkey anti-rabbit IgG conjugated to FITC (1:200) and goat anti-mouse IgG conjugated to Alexa Fluor 546 (1:200). 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. 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 \(\mu\)g/mL, aprotinin 30 \(\mu\)L/mL, leupeptin 1 \(\mu\)g/mL, and okadaic acid 10 \(\mu\)mol/L. Lysate proteins (20 \(\mu\)g) 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 \(\mu\)g/mL RNase (Oncogene Research Products) and 10 \(\mu\)g/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 (Multi-cycle, 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. 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 segment was grafted between the two ends of the carotid artery by sewing 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 \(\pm\) 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. 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 and Figure 2D). 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/H11002/H11002 cells but of WT cells (15±1.7 versus 28±2.4 of cells in S/G2-phase, respectively; n=4, *P<0.01; Figure 3D). However, the proliferative response to 10% FCS was not impaired in cav-1/H11002/H11002 VSMCs compared with their WT littermates (36.5±3.5 versus 32.1±2.8, cells in S/G2-phase; n=3, P=NS; Figure 3D). Notably, the basal proliferative rate of cav-1/H11002/H11002 cells was determined to be higher than that of WT cells (n=3, P<0.01, Figure 3D).

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. Src family kinases (SFKs) have also been shown to be involved in Akt activation. 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).
To further investigate the role of SFKs in PI3-K/Akt activation and to specifically probe for the involvement of c-Src in stretch-induced PI3-K activation, we transiently overexpressed a kinase-inactive mutant of c-Src (c-Src(K297R)) in rat VSMCs. Overexpression of c-Src(K297R) but not of a control vector expressing green fluorescent protein (GFP) prevented stretch-induced Akt activation (Figure 5C). The cells had been cotransfected and MACS-sorted before Western blotting to ensure analysis of a highly enriched, positively transfected cell population expressing the inactive c-Src mutant.

**Integrin αvβ3 Mediates Stretch-Induced PI3-K/Akt Activation**

We previously demonstrated that mechanosensitive PI3-K activation is dependent on an intact integrin/extracellular matrix interaction, and αvβ3 integrins have been shown to mediate stretch-induced VSMC proliferation. Therefore, experiments were performed to elucidate the role of specific integrins, in particular αvβ3 integrins, in mechanosensitive PI3-K activation. Lysates of cells stretched for 10 minutes were immunoprecipitated with anti–PI3-K (p85), and immunoblots were performed for αv and 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 C57/BL6j 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 6A). 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

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**Figure 5.** c-Src mediates stretch-induced PI3-K/Akt activation. A, VSMCs were exposed to cyclic stretch for 15 minutes. PP1 (10 μmol/L) was added 30 minutes before stretch. Mitogen-free, quiescent cells served as a control. PI3-K activity was determined using precipitated PI3-K of cellular lysates. B, Cells were exposed to cyclic stretch for 15 minutes. PP1 (10 μmol/L) was added 30 minutes before stretch. Akt phosphorylation was determined by immunoblotting. Cdk4 served as a control for equal protein loading. C, Cells were transfected with a kinase-inactive c-Src (c-Src[−]) or a control vector expressing only GFP 24 hours before positive selection by MACS. Positively transfected cells were then plated and exposed to 15 minutes of cyclic stretch.

We subsequently examined whether SFKs are involved in stretch-induced PI3-K/Akt activation. Previously, we had shown that Akt phosphorylation, under conditions of cyclic stretch, was strictly dependent on PI3-K activation because PI3-K inhibition (LY294002 and Wortmannin) completely prevented Akt phosphorylation. The addition of PP1, a specific inhibitor of the Src kinase family, not only prevented PI3-K activation (Figure 5A) but was also able to completely prevent stretch-induced Akt phosphorylation (Figure 5B), indicating that the SFK acts upstream of PI3-K.
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 null mouse into the carotid artery of a WT mouse allowed us to examine the effect of force on the cav-1 null vasculature independent of systemic limitations inherent in caveolin-1 gene-disrupted mice. In cav-1 null veins, Akt activation was completely abolished. Nevertheless, veins of cav-1 null 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 null 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 mechani-
cally triggered proliferation. Indeed, pharmacological inhibi-
tion of Erk during stretch did not impair the proliferative re-
response of VSMCs in vitro. Furthermore, our study also
points toward a predominant role of caveolin-1 for mecha-
nosensitive cell cycle entry and progression. Growth factor–
induced proliferation, however, was not impaired when
caveolin-1 was disrupted, a finding previously described.19

In vivo, the net effect of growth factors and mechanically
trigged stimuli determine the amount of neointima formation.
This may explain why late neointima formation was signifi-
cantly 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.20 Obviously, more factors have to be consid-
ered. In cav-1−/− mice, endothelial nitric oxide synthase (eNOS)
is activated.21 eNOS gene transfer has been shown to prevent
neointima formation in denuded rat carotid arteries.22 Therefore,
augmented endothelial nitric oxide synthesis may be addition-
ally responsible for reduced neointima formation in our in vivo
model as well as reduced plaque formation in ApoE−/− mice
interbred with cav-1−/−.23

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.19,24 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,25 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.26 The PDGF and the insulin
receptors also seem to initiate their signal transduction from
caveolae.27 Our data support the hypothesis that in the vascular-
ture, 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.28

In this study, we also demonstrate that cholesterol depletion of
the plasma membrane by β-cyclohextrin prevents PI3-K/Akt activ-
aton. 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.29 Consistent with our re-
results, reduction of plasma membrane cholesterol levels with cho-
lesterol-binding agents has been shown to inhibit specific agonist-
stimulated signaling pathways, eg, insulin-stimulated insulin
receptor substrate-1 phosphorylation29 or shear stress,30 and nerve
growth factor–induced31 and endothelin-1–induced32 activation of
the extracellular signal-regulated kinase, Erk. Although our data
suggest that caveolin-1 acts directly through protein-protein inter-
actions, 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,7,33 implying a re-
quirement 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 αβi integrins, but not βi integrins.34 In accordance with these findings, we find a
dependency of tensile stress-mediated PI3-K/Akt activation on
αβi integrins. Furthermore, Src family kinases are
required for the force-dependent formation of focal adhesion
complexes and strengthening of αβi integrin-cytoskeleton
connections.35 In addition, an involvement of Src family
kinases in PI3-K and in Akt activation has been reported before.36 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 αβi 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 prolifera-
tive disease processes and may have novel implications for
the future design of therapeutic interventions.

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References

1. Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and
atherosclerosis: new perspectives and therapeutic strategies. Nat
791–804.
3. Lehoux S, Tedgui A. Signal transduction of mechanical stresses in the
4. Sedding DG, Seay U, Fink L, Heil M, Kummer W, Tillmanns H, Braun-
Dullaeus RC. Mechanosensitive p27Kip1 regulation and cell cycle entry
5. Aplin AE, Howe A, Alahari SK, Juliano RL. Signal transduction and
signal modulation by cell adhesion receptors: the role of integrins, cad-
herins, immunoglobulin-cell adhesion molecules, and selectins.
13. Li S, Seitz R, Lisanti MP. Phosphorylation of caveolin by src tyrosine
15. Hanke JH, Gardner JP, Dow RL, Changelian PS, Wringier
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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<sup>k</sup>-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<sup>k</sup>-II MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Transfected (K<sup>k</sup>-II positive) cells were then separated on MS<sup>+</sup>/RS<sup>+</sup> 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<sup>k</sup>-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-<sup>32</sup>P]ATP, 20 µmol/l ATP, and 20 mmol/l MgCl<sub>2</sub> 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 dithiothreitil), 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.

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