Mechanosensitive $p27^{Kip1}$ Regulation and Cell Cycle Entry in Vascular Smooth Muscle Cells

Daniel G. Sedding, MD; Ulrike Seay; Ludger Fink, MD; Matthias Heil, PhD; Wolfgang Kummer, MD; Harald Tillmanns, MD; Ruediger C. Braun-Dullaeus, MD

Background—Cyclic stretch plays an important role in the homeostasis of vessel structure. Increased forces might, however, contribute to remodeling processes, resulting in vascular proliferative diseases. The initial molecular events necessary for mechanosensitive cell cycle entry of quiescent smooth muscle cells are poorly understood.

Methods and Results—In this study, we demonstrate that mechanical strain resulted in a rapid, integrin-dependent but mitogen-independent activation of phosphoinositide 3-kinase (PI3-K)/protein kinase B (Akt) in quiescent vascular smooth muscle cells. Subsequently, downstream ALL 1 fused gene from chromosome X (AFX)-like forkhead transcription factors were inactivated, leading to transcriptional downregulation of $p27^{Kip1}$. This contrasted with the posttranscriptional protein reduction of $p27^{Kip1}$ in cells stimulated with serum mitogens. Stretch-mediated $p27^{Kip1}$ downregulation was accompanied by activation of cyclin-dependent kinase 2, hyperphosphorylation of retinoblastoma protein, and proliferation. Forkhead transcription factor inactivation and $p27^{Kip1}$ downregulation were prevented by the PI3-K inhibitors wortmannin and LY294002. Pharmacological blockade of other kinases, such as p42/44, p38, and protein kinase A or C, did not influence the mechanosensitive gene regulation. $p27^{Kip1}$ downregulation and cell cycle entry were, however, prevented by overexpression of a constitutively inactive form of Akt or constitutively active forms of forkhead transcription factors.

Conclusions—Our data demonstrate that the earliest cell cycle events can occur in a solely mechanosensitive fashion. Vascular smooth muscle cells are, furthermore, able to use transcriptional or posttranscriptional mechanisms to regulate $p27^{Kip1}$, depending on the stimulus to which they are exposed. This observation has novel implications for understanding of vascular proliferative diseases. **(*Circulation*. 2003;108:616-622.)**

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

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In the vasculature, cells are constantly exposed to alternating mechanical forces, and the cell cycle plays an enormous role in maintaining vessel structure and allowing its adaptation to acute and chronic changes. Through these remodeling processes, altered mechanical forces can contribute to pathological changes of the vessel by inducing vascular smooth muscle cell (VSMC) migration, proliferation, and hypertrophy, which have been considered key events in the development of atherosclerosis, postangioplasty restenosis, and venous bypass graft failure.[1–3] Although signaling pathways responsive to mechanical force have come to light,[1,4] the initial molecular events necessary for mechanosensitive cell cycle entry of quiescent VSMCs are poorly understood.

The cyclin-dependent kinase (Cdk) inhibitor $p27^{Kip1}$ plays a critical role in the entry of quiescent, G_0 phase cells into the cell cycle.[5] Being present at maximal levels in quiescent cells, the amount of $p27^{Kip1}$ decreases as cells are stimulated to enter the cell cycle, thereby allowing the activation of G_1 phase Cdk/cyclin complexes with subsequent cell cycle progression.

Although the growth factor–induced decline in the amount of $p27^{Kip1}$ had first been demonstrated to occur solely through posttranscriptional processes, such as increased degradation or decreased protein synthesis,[6,7] it has recently become evident that downregulation of $p27^{Kip1}$ can also occur at the transcriptional level. AFX-like forkhead transcription factors were found to be involved in $p27^{Kip1}$ gene transactivation.[8] Furthermore, ALL 1 fused gene from chromosome X (AFX)-like forkhead transcription factor phosphorylation by phosphoinositide 3-kinase (PI3-K)–activated protein kinase B (Akt) resulted in their inactivation.[9] It is, however, not clear whether cells use transcriptional or posttranscriptional mechanisms of $p27^{Kip1}$ downregulation differentially, depending on the stimulus that leads to cell cycle entry.

With the aim of elucidating the earliest mechanosensitive cell cycle events, we were able to demonstrate that mechanical forces rapidly activate Akt in quiescent, serum-deprived, VSMCs, leading to AFX-like forkhead transcription factor inactivation. This event resulted in the transcriptional down-

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regulation of $p27^{Kip1}$ and cell cycle entry, without a change in the rate of its protein degradation. Activation of this signaling cascade was dependent on extracellular matrix/integrin interactions and independent of acutely released or newly synthesized growth factors. Our finding contrasts with the mitogen-induced cell cycle entry of VSMCs, which predominantly depends on increased $p27^{Kip1}$ protein degradation without a change in its mRNA level. Our data not only demonstrate the early mechanosensitive cell cycle regulation but also indicate that vascular cells are able to translate mechanical forces differentially into a signal toward cell cycle entry.

Methods

Reagents

The following antibodies were used: rabbit polyclonal anti-pAkt (Ser 473), anti-pFKHR (Thr 247), anti-pFKHRL1 (Thr 32), anti-pan–Akt1, and anti-phistone H3 (Ser 10) (all from New England Biolabs, Frankfurt, Germany). Mouse monoclonal anti-$p27^{Kip1}$, anti-retinoblastoma (RB), anti-Cdk2 (Santa Cruz Biotechnology, Santa Cruz, Calif), anti-basic fibroblast growth factor (bFGF), anti-insulinlike growth factor (IGF), and anti–platelet-derived growth factor (PDGF) (R&D Systems, GMBH, Weisbaden, Germany) were also used. Secondary antibodies included the following: goat anti-rabbit immunoglobulin G and goat anti-mouse immunoglobulin G, both of which were linked to horseradish peroxidase (Santa Cruz Biotechnology Inc), Arg-Gly-Asp (GRGDSP), and Arg-Gly-Asp control peptide (GRGESP) (both from Bachem Biochemica, Heidelberg, Germany). Wortmannin was obtained from Sigma (Diesenhofen, Germany); LY294002, from Biolmol (Plymouth Meeting, Pa); and PD98059, SB203580, KT5720, staurosporine, and the tyrphostines AG1478, AG1296, and AG1024, from Calbiochem (San Diego, Calif). 5-Bromo-2′-deoxyuridine (BrdU) and the anti-BrdU staining kit were obtained from Zymed (San Francisco, Calif).

Cell Culture, Stretch Apparatus, and Experimental Conditions

Primary cultures of VSMCs were initiated as previously descibed.[10] The cells were seeded (≈10 000 cells/mL) onto 6-well, fibronectin-coated plates (FlexI plates, Flexercell). Studies were conducted on VSMCs (passage 7 to 12) after they had achieved confluence in 10% fetal bovine serum/Dulbecco's modified Eagle's medium/Ham's F12 medium, followed by serum withdrawal for 2 days to achieve quiescence. On the day of the experiment, fresh, serum-free medium was substituted, and cyclic stretch was applied with a commercially available apparatus (125% resting length, 0.5 Hz; Flexercell) in a tissue-culture incubator.

Preparation of Cellular Lysates, Immunoblot Analysis, and Histone H1 Kinase Assay

Specific protein content in the cell lysates was analyzed by immunoblot, as previously described.[10] In brief, the supernatant was run on a polyacrylamide gel and blotted onto nitrocellulose (Hybond-ECL, Amersham) by wet electroblotting. After being blocked, the blots were incubated with primary antibody (1:1000 dilution for anti-pAkt; 1:500 for anti-pFKHR; 1:500 for anti-pFKHRL1; 1:2000 for anti–pan-Akt1; 1:200 for anti-$p27^{Kip1}$; and 1:100 for anti-RB) for 1 hour at room temperature. Specific proteins were then detected by enhanced chemiluminescence (ECL+, Amersham) after being labeled with horseradish peroxidase–labeled secondary antibody (1:2000 for 1 hour) according to the manufacturer's instructions.

For the histone H1 kinase assay, lysates were labeled with an anti-Cdk2 antibody (1 μg/250 μg protein), and immune complexes bound to protein A/G–agarose beads (Oncogene Sciences) were assayed by addition of kinase buffer, histone H1, and [^{32}P]ATP (3000 Ci/mmol, DuPont–New England Nuclear), as described previously.[10] The samples were boiled for 5 minutes, electrophoresed through a 12% sodium dodecyl sulfate–polyacrylamide gel, dried, and exposed to x-ray film.

Transfection Procedure and Plasmids

For overexpression studies, a commercially available lipid formulation (Fugene, Roche) was used. For a 35-mm dish, 6 μL liposomes were added to 100 μL Opti-MEM (GIBCO BRL) and mixed with 2 μg DNA of either plasmid before being added to the mixture with the cells, resulting in a 20% to 30% transfection efficiency. Successfully cotransfected cells were subsequently selected by magnetic-activated cell sorting (MACS; see following section). npAkt was kindly donated by K. Walsh, Tufts University School of Medicine, Boston, Mass; pECE.FKHR-L1 and pECE.FKHR-L1.A3 by M. Greenberg, Harvard, Boston, Mass; and pBabe, AFX, and pMT2HA-AFX.A3 by B.M.T. Burgering, University Medical Center, Utrecht, the Netherlands.

Analysis of Proliferation

For flow cytometric analysis, cells were harvested by trypsinization, fixed overnight with 75% methanol, washed, and incubated with 100 μg/mL RNase (Oncogene) and 10 μg/mL propidium iodide in phosphate-buffered saline for 1 hour at 37°C. Samples were analyzed for DNA content with a high-speed cell sorter (EPICs Altra, Beckman Coulter), and data were analyzed by computer with commercially available software (Multicycle, Phoenix Flow Systems).

For additional determination of DNA synthesis, 10 mmol/L BrdU was added to the medium, and cells were stretched for 24 hour. After fixation, BrdU-positive nuclei were quantified after visualization with a monoclonal anti-BrdU antibody and subsequent diaminobenzidine staining, according to the manufacturer's instructions (Zymed). VSMCs in mitosis were quantified after immunocytochemical staining for phosphorylated histone H3 and subsequent fluorescence microscopy (Cell Signaling).

Real-Time RT-PCR

Relative mRNA quantification was performed with the Sequence Detection System 7700 (PE Applied Biosystems) and real-time reverse transcription–polymerase chain reaction (RT-PCR). By applying comparative quantification, the target gene was normalized to an internal-standard gene, as described before.[11,12] For internal calibration, mRNA transcribed from the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. Amplification efficiency of GAPDH and $p27^{KIP1}$ primer/probe sets were approximately equal and amounted to 1.0 (=100%).

For cDNA synthesis and real-time RT-PCR, reagents, primers, and probes were applied as described before.[12] The sequences, amplicon sizes, and exon localization of primers and probes used were as follows: GAPDH (amplicon size, 121 bp): GAPDH forward, 5′-GTGATGGGTGTGAACCACGAG-3′ (exon e5), and GAPDH reverse, 5′-CCACGATGCCAAAGTTGTCA-3′ (exon e6); GAPDH-hybridization probe: 5′-CTCAAGATTGTCAGCAATGCA-TCCTGCAC-3′ (exon e5–e6); $p27^{Kip1}$ (amplicon size, 75 bp): $p27^{Kip1}$ forward, 5′-GCAGTGTCCAGGGATGAGGA-3′(exon e1); $p27^{Kip1}$ reverse, 5′-TCTGTTCTGTTGGCCCTTTTGT-3′ (exon e2); and $p27^{Kip1}$ hybridization probe, 5′-ACCTGCGGCAGAAGATT-CTTCTTCGC-3′ (exon e1–e2).

Magnetic-Activated Cell Sorting

Cells were cotransfected by using equimolar amounts of pMACS.K^k-II and an expression plasmid as indicated. After 24 hours, transduced cells were trypsinized and magnetically labeled with MACSelect K^k-II MicroBeads (Miltenyi Biotec). Transfected (K^k-II–positive) cells were then separated on an MS^+/RS^+ separation columns (Miltenyi Biotec) and subsequently lysed in the appropriate buffer. Positively selected cells were >85% positive for K^k-II expression.

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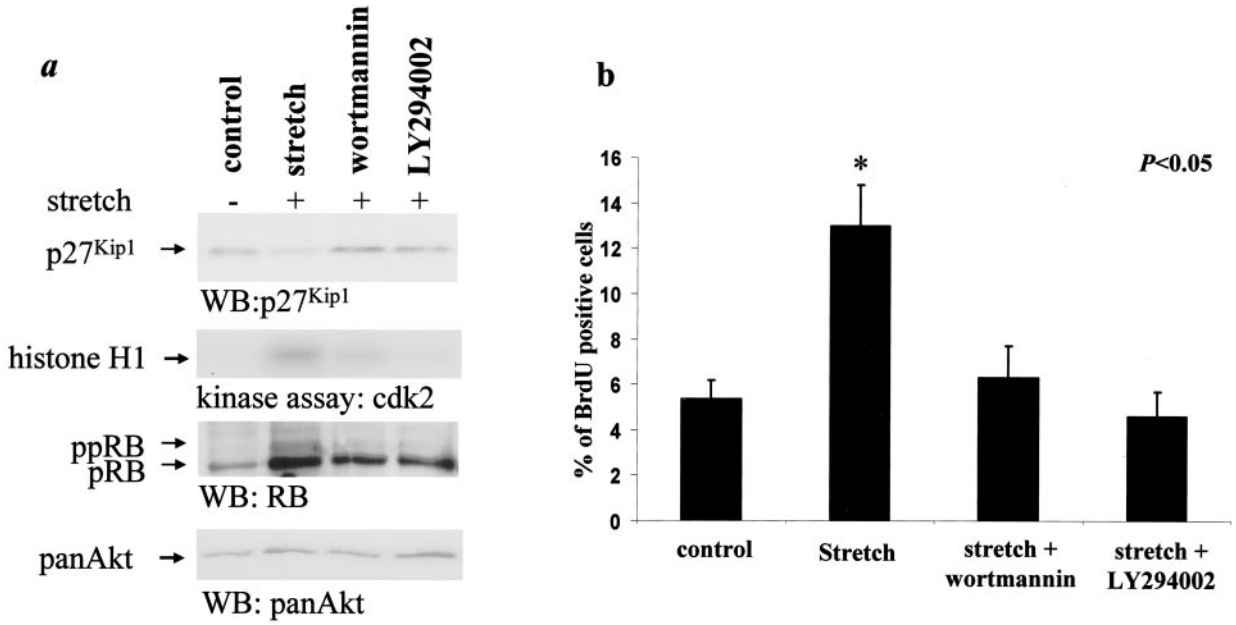

Figure 1. Cyclic strain initiates $p27^{Kip1}$ downregulation and cell cycle entry of quiescent VSMCs. Cyclic stretch was applied for up to 24 hours (125% resting length, 0.5 Hz). a, $p27^{Kip1}$ and RB were examined by immunoblotting (WB) after 24 hour of cyclic stretch. Shift indicates hyperphosphorylation of RB. Cdk2 activity was determined by histone H1 kinase assay. Pan-Akt served as housekeeping protein. b, Quantification of BrdU-positive cells (n=4). *$P<0.05$, addition of PI3-K-inhibitors wortmannin and LY294002 before cyclic stretch significantly inhibited VSMC proliferation compared with stretch cells.

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 3 times.

Results

Cyclic Stretch Results in Cell Cycle Entry of VSMCs

After subjecting quiescent VSMCs to cyclic stretch (24 hours, 125% resting length, 0.5 Hz), we observed a rapid downregulation of $p27^{Kip1}$ within 10 to 12 hours, which reached a nadir after 24 hours (data not shown). $p27^{Kip1}$ downregulation was accompanied by enzymatic activation of Cdk2 and hyperphosphorylation of the RB protein, indicating cell cycle entry (Figure 1a). Indeed, 24 hours of cyclic stretch resulted in a decline of VSMCs in the G_0/G_1 phase, from 93.1±1.8% to 83.4±1.4% ($P<0.01$), similar to the 1.9-fold increase in the number of proliferating cells, as quantified by fluorescence-activated cell sorting of propidium iodide–stained VSMCs, which indicated entry into the cell cycle (Figure 3b). In additional, BrdU incorporation had significantly increased (Figure 1b). Furthermore, cell cycle progression through mitosis was quantified by fluorescence staining for phosphorylated histone H3. The number of cells positive for this marker increased significantly within 24 hour of cyclic stretch (control, 2.6±0.8%; stretched, 8.4±1.4%; n=3, $P<0.05$).

Inhibition of PI3-K/Akt Stabilizes $p27^{Kip1}$ and Prevents Stretch-Induced Cell Cycle Entry

Strain-induced downregulation of $p27^{Kip1}$ protein levels was prevented when PI3-K/Akt signaling was inhibited with the specific PI3-K inhibitors wortmannin or LY294002. Accordingly, activation of Cdk2 was prevented, RB remained hypophosphorylated (Figure 1a), and cell cycle entry was blocked (Figures 1b and 3b). Also, the stretch-induced increase in the number of cells positive for phosphorylated histone H3 was prevented (control, 2.6±0.8%; stretched, 8.4±1.4%; stretched+wortmannin, 3.2±1.0%; and stretched+LY294002, 2.8±0.8%; n=3, $P<0.05$). Similar data were obtained when a nonphosphorylatable, inactive form of Akt (npAkt) was transiently overexpressed (Figures 2a and 3b). Conversely, we were unable to stabilize $p27^{Kip1}$ through inhibition of other central signaling cascades, such as p42/44 and p38 mitogen-activated protein kinase, protein kinase A, or protein kinase C, by using their specific inhibitors PD98059, SB203580, KT5720, or staurosporine, respectively (Figure 2b).

Cyclic Strain–Activated Akt Mediates AFX-Like Forkhead Transcription Factor Phosphorylation, Resulting in $p27^{Kip1}$ Transcriptional Repression

Cyclic strain of quiescent VSMCs resulted in rapid and marked phosphorylation of Akt, (reaching maximal levels at 10 to 15 minutes and decreasing thereafter), as it did during serum stimulation, although serum seemed more potent (data not shown). Forkhead transcription factors, which represent a direct downstream target of Akt,[9] were found to be phosphorylated and thereby inactivated (Figure 2c). Either state was prevented by inhibition of PI3-K (wortmannin and LY294002) but not by inhibition of other signaling cascades (only the results of p42/44 and p38 inhibition by PD98059 and SB203580, respectively, are shown).

We subsequently used overexpression studies to further characterize the involvement of AFX-like forkhead transcrip-

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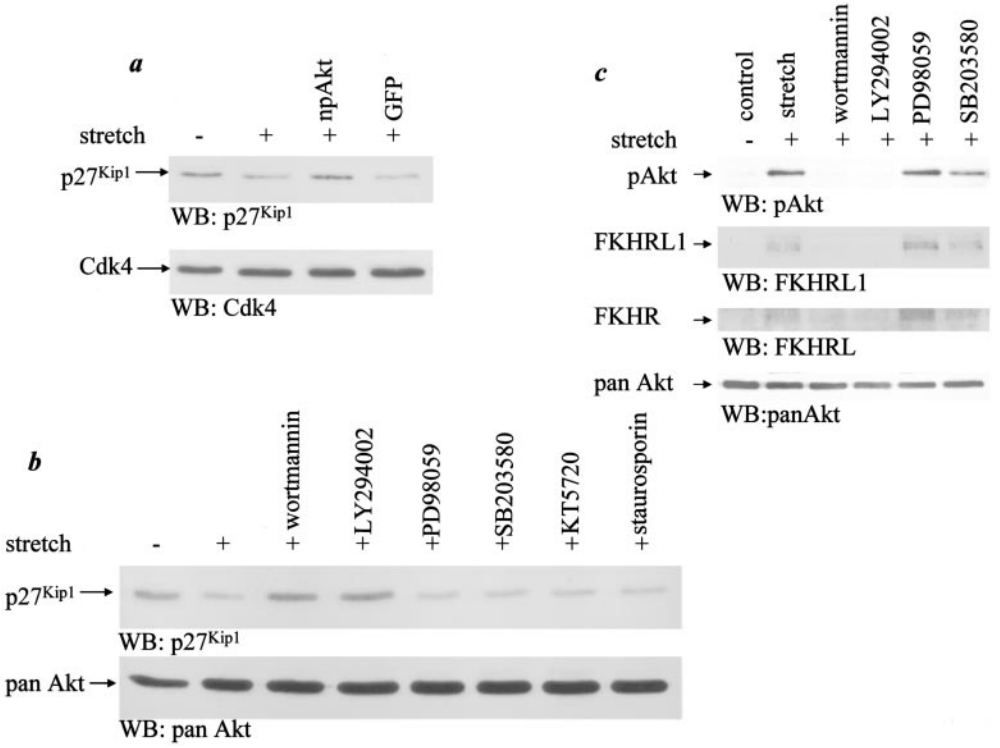

Figure 2. Stretch-induced downregulation of $p27^{Kip1}$ is mediated through PI3-K and activated Akt. a, Nonphosphorylatable, inactive form of Akt (npAkt) was cotransfected with equimolar amount of pMACS.K^k-II vector. After 24 hours of stretch, cells were selected by using MACS column, and $p27^{Kip1}$ protein levels were analyzed by immunoblotting (WB). Cotransfection with pCDNA.3 vector containing insert coding for green fluorescent protein (GFP) served as control. Cdk4 served as housekeeping protein. b, Cells were treated with specific inhibitors for PI3-K (wortmannin, LY294002), p42/44 (PD98059), p38 MAPK (SB203580), protein kinase A (KT5720), and protein kinase C (staurosporine) before 24 hours of cyclic stretch. $p27^{Kip1}$ protein levels were analyzed by immunoblotting (WB). Pan-Akt served as housekeeping protein. c, Cells were exposed to cyclic stretch for 15 minutes and phosphorylation of Akt, FKHRL1, and FKHR was assessed by immunoblotting (WB) with antibodies specific for their phosphorylated proteins. Inhibitors of PI3-K and MAPKs had been added 30 minutes before stretch. Pan-Akt served as housekeeping protein.

tion factors in strain-induced $p27^{Kip1}$ downregulation. Overexpression of a nonphosphorylatable, constitutively active form of FKHRL1 (FKHRL1.A3) or AFX (AFX.A3), but not a control vector (GFP), prevented the stretch-induced reduction of $p27^{Kip1}$ protein levels (Figure 3a), resembling those seen in quiescent VSMCs. Furthermore, overexpression of FKHRL1.A3 and AFX.A3 prevented cell cycle entry of stretched cells (Figure 3b). When we used wild-type AFX or FKHRL1 for overexpression studies, $p27^{Kip1}$ levels were only restored partially, pointing toward its intact regulation during stretch.

Cyclic Strain Alters $p27^{Kip1}$ Gene Transcription but Not Its Protein Degradation

Because growth factor–induced downregulation of $p27^{Kip1}$ had been demonstrated to principally occur through posttranscriptional processes,[6,7] we analyzed its degradation kinetics during cyclic stretch and compared it with the kinetics of quiescent cells and cells subjected to serum stimulation (Figure 4). We determined a half-life of 40 ± 6 minutes for $p27^{Kip1}$ in quiescent VSMCs, which was reduced to 17 ± 8 minutes ($P<0.05$, $n=3$) when the cells were exposed to serum, demonstrating increased degradation. However, cyclic stretch did not change the degradation kinetics of $p27^{Kip1}$ at all (half life, 38 ± 8 minutes) compared with quiescent cells (Figure 4). Conversely, cyclic stretch resulted in a rapid downregulation of $p27^{Kip1}$ mRNA levels, as quantified by real-time RT-PCR and compared with quiescent, nonstretched cells (Figure 5; $P<0.001$, $n=3$). These data demonstrate that both growth factors and mechanical strain result in $p27^{Kip1}$ downregulation, although through different mechanisms.

Soluble Factors Are Not Involved in Stretch-Induced Akt/Forkhead Transcription Factor Signaling

We further sought to identify the upstream mechanisms underlying the rapid strain-induced activation of the PI3-K/Akt signaling pathway. Mechanical stress results in synthesis and release of growth factors, such as IGF, PDGF, and bFGF, from VSMCs, which might activate several signaling cascades in an autocrine or paracrine manner.[13–15] We tested the involvement of these factors in mediating stretch-induced Akt activation and $p27^{Kip1}$ transcriptional downregulation by adding neutralizing antibodies against IGF, PDGF, or bFGF to the media before the cell stretching experiments. The effectiveness of these neutralizing antibodies in blocking the target molecules had been determined in preceding experiments (data not shown). None of these antibodies prevented Akt activation or $p27^{Kip1}$ downregulation during stretch (Figure

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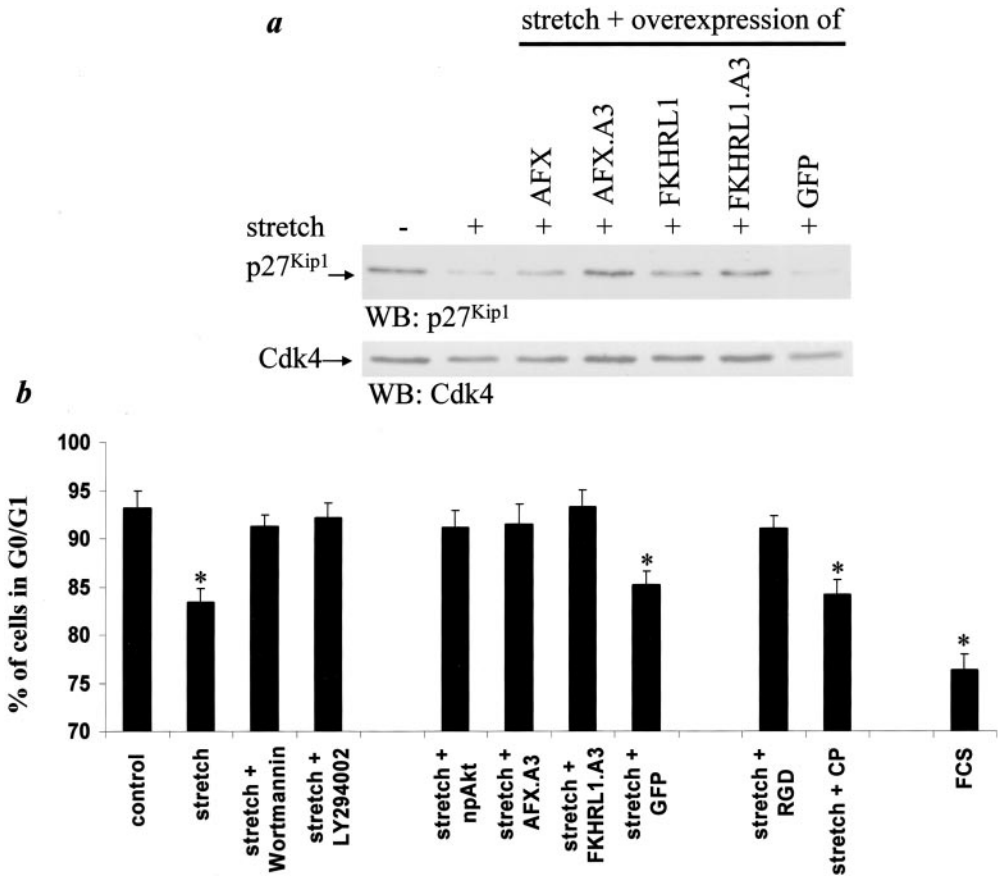

Figure 3. Overexpression of nonphosphorylatable, constitutively active forkhead transcription factors reconstitutes $p27^{Kip1}$ and prevents cell cycle entry of VSMCs exposed to cyclic stretch. a, Cells were cotransfected with wild-type (AFX and FKHRL1) or nonphosphorylatable, constitutively active AFX-like forkhead transcription factors (AFX.A3 and FKHRL1.A3) together with equimolar amount of pMACS.K^k-II vector. After exposure to cyclic stretch for 24 hours, cells were selected by using MACS column, and $p27^{Kip1}$ expression was assessed by immunoblotting (WB). pCDNA.3 vector containing insert coding for GFP served as control. Cdk4 served as housekeeping protein. b, Cell cycle distribution was determined by fluorescence-activated cell sorting analysis of propidium iodide–stained cells (shown is number of cells in G_0/G_1 phase under various conditions; n=4, $^*P<0.01$). CP indicates control peptide; FCS, fetal calf serum.

6a). Even applying all antibodies simultaneously did not affect this mechanosensitive signal transduction (data not shown). Furthermore, we examined the effect of conditioned medium collected from VSMCs after 15 minutes of cyclic stretch. Treatment of quiescent VSMCs with conditioned medium did not affect Akt phosphorylation (data not shown).

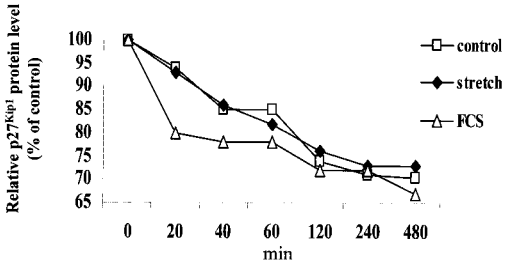

Figure 4. Degradation of $p27^{Kip1}$ protein is not altered when cells are exposed to cyclic stretch. Cycloheximide was added at t=0 minutes to prevent de novo protein synthesis, allowing determination of $p27^{Kip1}$ degradation kinetics. Cells were stretched for time intervals indicated, and $p27^{Kip1}$ protein levels were assessed by planimetry of immunoblots. Densitometric analysis (relative protein content of quiescent (□), stretched (♦), and serum-stimulated (Δ) cells. FCS indicates fetal calf serum.

To exclude a possible growth factor–independent but growth factor receptor–mediated activation of the PI3-K/Akt pathway, we added specific inhibitors of growth factor receptors, the tyrphostines AG1478 (EGF), AG1296 (PDGF), and AG1024 (IGF-1) to quiescent VSMCs before stretch. Neither stretch-induced Akt phosphorylation nor $p27^{Kip1}$ downregulation was prevented by these tyrphostines (Figure 6b). Our data indicate

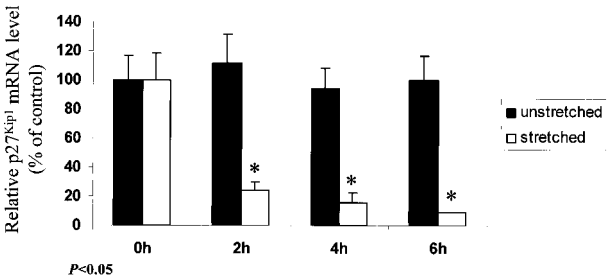

Figure 5. $p27^{Kip1}$ mRNA levels are rapidly downregulated in stretched VSMCs. Cells were stretched for intervals indicated, and $p27^{Kip1}$ mRNA levels were analyzed by quantitative real-time RT-PCR. All values are means of 3 independent determinations normalized to expression level of GAPDH. $^*P<0.05$ compared with unstretched control.

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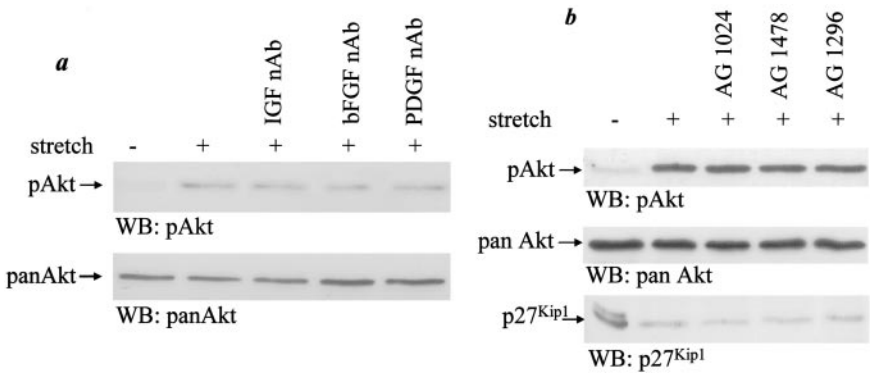

Figure 6. Stretch-induced Akt activation is growth factor and growth factor receptor independent. a, Neutralizing antibodies (nAb) against IGF, bFGF, or PDGF were added, and cells were stretched for 15 minutes. Phosphorylation of Akt was determined by immunoblotting (WB) with phosphospecific antibody. Pan-Akt served as housekeeping protein. b, Growth factor receptor inhibitors AG1478 (EGF), AG1296 (PDGF), and AG1024 (IGF-1) were added, and cells were stretched for 15 minutes or 24 hours to determine Akt activation and $p27^{Kip1}$ expression by immunoblotting (WB), respectively. Pan-Akt served as housekeeping protein.

that early stretch-induced Akt phosphorylation and later $p27^{Kip1}$ downregulation are not caused by a release of growth factors, growth factor receptor activation, or other soluble factors.

RGD Proteins Perturb the Ability of VSMCs to Sense Mechanical Strain

To test the involvement of extracellular matrix/integrin interactions, we examined whether the integrin-binding peptide RGD would interfere with the response of quiescent VSMCs to mechanical strain. Addition of RGD peptide to the medium before cyclic stretch dose-dependently blocked phosphorylation of Akt and prevented downregulation of $p27^{Kip1}$ (Figure 7), suggesting that an intact matrix/integrin interaction is critical for mechanosensitive $p27^{Kip1}$ downregulation by way of PI3-K/Akt/AFX-like forkhead transcription factor signaling. Consequently, addition of RGD peptide, but not control peptide, to quiescent VSMCs was able to prevent stretch-induced proliferation (Figure 3b).

Discussion

Mechanotransduction plays a critical role in vascular homeostasis. However, in contrast to growth factor–induced cell cycle entry and progression, not much is known about its direct influence on cell cycle regulation. Although moderate cyclic stretch, as occurs under physiological conditions, seems essential for maintaining the vessel wall structure and for inhibiting growth factor–stimulated proliferation of VSMCs,[16] increased stretch, more like that resembling pathological conditions as occurs in severe hypertension, in venous bypass grafts, or during balloon angioplasty, was reported to induce proliferation of VSMCs.[4] Whether mechanical force directly exerts influence on the initial events of cell cycle entry and whether this might be able to trigger the development of vascular proliferative diseases were, however, unclear. Our study provides evidence that VSMCs, when exposed to an enhanced mechanical force, use extracellular matrix/integrin interactions to activate Akt, which,, in turn,, phosphorylates and inactivates AFX-like forkhead transcription factors, leading to transcriptional repression of the G_0 phase gatekeeper, $p27^{Kip1}$. This chain of events is growth factor independent and initiates cell cycle entry and progression, as indicated by Cdk2 activation, RB hyperphosphorylation, and subsequent increased DNA synthesis and mitosis.

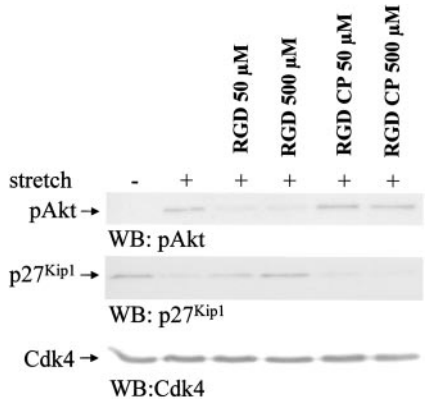

Figure 7. Cyclic strain–induced Akt activation and $p27^{Kip1}$ downregulation are dependent on intact extracellular matrix/integrin interaction. RGD peptide or inactive control peptide (CP) was added at indicated concentrations, and cells were stretched for 15 minutes or 24 hours to determine Akt phosphorylation and $p27^{Kip1}$ protein expression levels, respectively. Cdk4 served as housekeeping protein.

$p27^{Kip1}$ has primarily been demonstrated to be regulated by way of posttranscriptional processes (inhibition of synthesis or ubiquination and subsequent proteasome-dependent degradation) when growth factors are used to initiate cell cycle entry.[6,7] Indeed, serum-induced downregulation of $p27^{Kip1}$ in quiescent VSMCs resulted mainly from its protein degradation and furthermore, was independent of PI3-K/Akt signaling, because the inhibitors wortmannin or LY294002 did not result in stabilization of $p27^{Kip1}$ protein levels, a finding previously reported by us.[17] Conversely, extracellular matrix/integrin interactions, initiated by mechanical strain in the absence of growth factors, nearly exclusively resulted in the transcriptional repression $p27^{Kip1}$ due to inhibition of AFX-like forkhead transcription factor activity. Forkhead transcription factors have already been functionally implicated in the regulation of metabolism, cell proliferation, and apoptosis.[9] Recently, it has been demonstrated that they are directly phosphorylated and inactivated by Akt,[8] identifying them as additional targets of PI3-K/Akt signaling.

In our study, extracellular matrix/integrin interaction resulted in forkhead transcription factor–dependent transcriptional downregulation of $p27^{Kip1}$. The kinetic of the mRNA decrease, as well as the experiments with conditioned medium and neutralizing antibodies, implies independence from newly synthesized growth factors. Using tyrphostines, we were also able to rule out an involvement of growth factor

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receptors, which could have been activated independently of growth factors themselves.

Although we were able to dissect $p27^{Kip1}$ downregulation in vitro, it seems likely that vascular cells in vivo use both translational and posttranslational mechanisms of $p27^{Kip1}$ regulation during arterial remodeling. Simultaneously, a highly mitogenic milieu is locally created by pathological trauma to the vessel wall, as occurs in vascular proliferative diseases.[13,15] Synergy between mitogenic stimulation and mechanical force has been suggested before.[15,18,19] Our data, however, imply that the early steps of stretch-induced cell cycle entry might be triggered by mechanotransduction rather than mitogenic activation.

Protein kinase C and MAPKs have been shown to be activated in force-induced VSMC proliferation (for a review, please see Li and Xu[4]). Although we also determined rapid activation of extracellular signal–regulated kinase 1/2 when VSMCs were stretched in vitro (data not shown), its inhibition as well as that of p38 MAPK, protein kinase A, or protein kinase C did not result in stabilization of $p27^{Kip1}$, thus excluding involvement of these signaling pathways in its force-induced early downregulation.

Further in vivo studies will examine the role of mechanosensitive $p27^{Kip1}$ downregulation in vascular remodeling processes and the development of vascular proliferative diseases. Recent in vivo studies have demonstrated that apolipoprotein E/$p27^{Kip1}$ double-knockout mice develop enhanced proliferation of VSMCs and accelerated atherosclerosis. Interestingly, a very moderate decrease of $p27^{Kip1}$ expression levels ($p27^{-/+}$ mice) was sufficient to predispose to atherosclerosis.[20] Also, gene transfer of $p27^{Kip1}$ was able to inhibit neointima formation in the rat carotid artery.[21] Controversially, however, $p27^{Kip1}$-deficient mice did not show an altered arterial wall proliferative response to femoral artery transluminal injury.[22]

Mechanosensitive regulation of $p27^{Kip1}$ through PI3-K/Akt and AFX-like forkhead transcription factors might play an important role in physiological vascular remodeling processes and the pathophysiology of vascular proliferative diseases. Our observations have novel implications for the understanding of vascular proliferative disease processes.

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