The Mechanical Stress–Activated Serum-, Glucocorticoid-Regulated Kinase 1 Contributes to Neointima Formation in Vein Grafts

Jizhong Cheng, Ying Wang, Yewei Ma, Bonita Tak-yeey Chan, Min Yang, Anlin Liang, Liping Zhang, Huihua Li, Jie Du

Rationale: Mechanical stress plays an important role in proliferation of venous smooth muscle cells (SMCs) in neointima, a process of formation that contributes to failure of vein grafts. However, it is unknown what intracellular growth signal leads to proliferation of venous SMCs.

Objective: The objective of this study is to identify mechanisms of mechanical stretch on neointima formation.

Methods and Results: By a microarray analysis, we found that mechanical cyclic stretch (15% elongation) stimulated the transcription of SGK-1 (serum-, glucocorticoid-regulated kinase-1). Mechanical stretch–induced SGK-1 mRNA expression was blocked by actinomycin D. The mechanism for the SGK-1 expression involved MEK1 but not p38 or JNK signaling pathway. SGK-1 activation in response to stretch is blocked by insulin-like growth factor (IGF)-1 receptor inhibitor and mammalian target of rapamycin complex (mTORC)2 inhibitor (Ku-0063794) but not mTORC1 inhibitor (rapamycin). Mechanical stretch–induced bromodeoxyuridine incorporation was reduced by 83.5% in venous SMCs isolated from SGK-1 knockout mice. In contrast, inhibition of Akt, another downstream signal of PI3K resulted in only partial inhibition of mechanical stretch–induced proliferation of venous SMCs. Mechanical stretch also induced phosphorylation and nuclear exportation of p27kip1, whereas knockout of SGK-1 attenuated this effect of mechanical stretch on p27kip1. In vivo, we found that placement of a vein graft into artery increased SGK-1 expression. Knockout of SGK-1 effectively prevented neointima formation in vein graft. There is significant lower level of p27kip1 located in the nucleus of neointima cells in SGK-1 knockout mice compared with that of wild-type vein graft. In addition, we also found that wire injury of artery or growth factors in vitro increased expression of SGK-1.

Conclusions: These results suggest that SGK-1 is an injury-responsive kinase that could mediate mechanical stretch–induced proliferation of vascular cells in vein graft, leading to neointima formation. (Circ Res. 2010;107:1265-1274.)

Key Words: mechanical stretch ■ serum-, glucocorticoid-regulated kinase-1 ■ neointima formation ■ mTORC ■ mammalian target of rapamycin complex

Increased growth of venous smooth muscle cells (SMCs) is a hallmark of vein graft failure.1–4 After implantation of a vein graft into the arterial circulation, the venous wall is immediately exposed to mechanical stimuli. Those include arterial pressure, wall tension, shear stress, and pulsatile flow, all of which modulate graft physiology.5–7 Such alteration could lead to the activation of many intracellular signaling pathways. Mechanical stress could also stimulate the synthesis and/or secretion of various bioactive molecules including platelet-derived growth factor (PDGF), basic fibroblast growth factor, and the transcriptional factors.8–10 Recently, we have shown that mechanical stretch stimulates expression and activation of insulin-like growth factor (IGF)-1 and its receptor in venous SMCs, and we found that activated IGF-1R/phosphatidylinositol 3-kinase (PI3K) is essential for mechanical stretch–induced proliferation of venous SMCs.11 However, the downstream targets of IGF-1/PI3K signaling that mediate mechanical stretch–induced proliferation of venous SMCs are still unknown.

Belonging to the AGC subfamily, the catalytic domain of SGK-1 (serum-, glucocorticoid-regulated kinase-1) is 54% identical to that of AKT, and it shares common downstream substrates with AKT.12,13 Activation of SGK-1 or AKT needs phosphorylation of threonine at activation loop by PI3K/PDK1 and the serine at C-terminal hydrophobic motif by mammalian target of rapamycin complex C2 (mTORC2).14–17 SGK-1 has been reported to inhibit apoptosis in other cell types, especially in tumor cells. SGK-1 also illustrated its role in transcriptional inhibition of a proapoptotic factor of the
Model of Primary Mouse Venous SMCs Subjected to Cyclic Stretching to Determine Possible Mechanosensitive Mechanism for Neointima Formation in Vein Graft, we used an in vitro experimental system of mechanical stretch–treated venous SMCs.

Expression Both in the Neointima of a Vein Graft

Statistical Analysis

Method

Microarray Analysis

RNA was extracted by use of a Qiagen RNeasy kit (Qiagen, Valencia, Calif). Labeling, hybridization, washing, scanning, and initial analysis were performed by the Baylor College of Medicine Microarray Core Facility using standard Affymetrix protocols and an Affymetrix genome 430 2.0 array. Four chips were analyzed from 2 independent pools of control and stretched smooth muscle cell. Affymetrix CEL files were imported into GeneSpring GX (Agilent, Santa Clara, Calif), and GC robust multiarray average was used to perform background correction and normalization. Differential gene expression was filtered using the volcano plot filter in GeneSpring, with a probability value of < 0.001 and a minimum two fold change. This resulted in 714 differentially expressed probe sets, which, by taking into account multiple probe sets for the same gene, corresponded to 508 genes upregulated at least 2-fold and 206 genes downregulated at least 2-fold.

Statistical Analysis

All data are presented as means ± SEM. Comparison between groups was made using 1-way ANOVA; *P < 0.05 was considered statistically significant.

Results

There is a Significant Increase in the SGK-1 Expression Both in the Neointima of a Vein Graft and in Mechanical Stretch–Treated Venous SMCs

To determine possible mechanosensitive mechanism for neointima formation in vein graft, we used an in vitro experimental model of primary mouse venous SMCs subjected to cyclic stretch. Venous SMCs were subjected to 15% cyclic stretch (1 Hz) for 2 hours; unstretched cells were served as controls.

Microarray was performed to determine the stretch sensitive genes. There are total of 514 genes were regulated significantly (≥ 2-fold) after mechanical stretch. The heat map in Figure 1A showed some highly upregulated genes in this experiment. Among them, SGK-1 has a 9-fold increase. To confirm that SGK-1 is indeed expressed in vein graft, arterialized veins were collected from patient and SGK-1 staining was observed in the nucleus of neointima cells (Figure 1B). The increase in SGK-1 was also observed in mouse vein graft model. As shown in Figure 1C, a large number of SGK-1–positive (brown) smooth muscle cells and endothelial cells were found in sections of the neointima of vein graft. High-resolution staining indicated that most of SGK-1 signals are present in the nucleus of venous SMCs (Figure 1C, bottom). To determine whether SGK-1 mRNA is also increased in vein graft, total RNA was collected from control, vena cava vein and from vein graft 1 month after surgery, real-time RT-PCR was performed. As shown in Figure 1D, the SGK-1 mRNA was increased 6.2 ± 0.9-fold compared with the SGK-1 level in control vein. The level of SGK-1 mRNA was further detected in stretch-treated venous SMCs. As shown in Figure 1E, mechanical stretch induced a 8.8 ± 1.0-fold increase in SGK-1 mRNA at 2 hours in cultured venous SMCs (P < 0.01). There was a second peak at 12 hours after mechanical stretch treatment. The protein level of SGK-1 in stretch-treated venous SMCs was increased in a time-dependent fashion and peaked after 4 hours, it lasted 12 hours before return to normal level at 16 hours (Figure 1F, P < 0.01). To determine whether mechanical stretch–induced increases in SGK-1 mRNA was transcriptional, venous SMCs were pretreated with actinomycin D, the effect of stretch on SGK-1 expression was examined. As shown in Figure 1G, actinomycin D effectively blocked stretch-induced increases in SGK-1 mRNA. Furthermore, the SGK-1 promoter analysis showed that stretch significantly stimulated SGK-1 promoter activity (Figure 1H). To determine whether stretch increases SGK-1 kinase activity, we used GST-GSK3 as a substrate and found that SGK-1 kinase activity was increased in a time-dependent fashion and peaked after 4 hours, it lasted 12 hours before return to normal level at 16 hours (Figure 1I). It has been shown that activated SGK-1 is translocated into the nucleus, and we found that stretch-stimulated SGK-1 nuclear translocation (Figure 1J). Taken together, SGK-1 gene expression and activation increased in response to mechanical stretch.

MEK1 Signaling Pathway Mediates Mechanical Stretch–Induced SGK-1 Expression

Mitogen-activated protein kinase (MAPK) signaling pathway has been reported to manipulate SGK-1 expression. Mechanical stretch stimulated strong phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and p38, and moderate phosphorylation of c-Jun N-terminal kinase (JNK) (Figure 2A). We then inhibited different MAPKs in venous SMC and found that inhibition of MEK1 with inhibitor (U0126) or MEK1 dominant negative, but not inhibitor of p38 or JNK, significantly inhibited stretch-induced increases in SGK-1 protein level (Figure 2B and 2C). Similarly, the stretch induced SGK-1 promoter activities were also blocked by dominant negative form of MEK1, but not by MKK6 or JNK. These results suggested that MEK1 mediates stretch-induced SGK-1 expression. Because mechanical stretch also

Non-standard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>BrdUrd</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>IGF-1R</td>
<td>insulin-like growth factor-1 receptor</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mTORC2</td>
<td>mammalian target of rapamycin complex 2</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>SGK-1</td>
<td>serum-, glucocorticoid-regulated kinase</td>
</tr>
<tr>
<td>SMA</td>
<td>smooth muscle actin</td>
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It is known that expression of SGK-1 can be induced by several stress conditions, such as oxidative stress, hyperglycemia, and exposure to hyperosmolarity or steroid hormones. The aim of this study was to determine the signaling pathways that are responsible for mechanical stretch–induced SGK-1 expression and activation.
stirulates signals such as PI3K$^{24}$ and Egr-1,$^{11}$ we determined their roles in mechanical stretch–regulated expression of SGK-1. PI3K inhibitor (Ly294002) did not inhibit stretch-induced increases in both SGK-1 mRNA as shown in Figure 2E. Moreover, knockout Egr-1 had no effect on stretch-stimulated SGK-1 expression (Figure 2F). To provide an initial insight into the potential role of SGK-1 expression in general vascular remodeling, we also examined the SGK-1 expression in wire injury model and found that wire injury increased SGK-1 in injured artery (Figure 2G). Because growth factors are involved in the process of neointima formation, we examined if growth factors can increase expression of SGK-1. We found that treatment of venous SMCs with IGF-1, PDGF, or angiotensin II significantly increased SGK-1 level (Figure 2H). The PDGF-BB- or angiotensin II-induced increases in SGK-1 level were blocked by MEK1 inhibitor, but not by p38 or JNK inhibitors. Interestingly, the IGF-1 induced-SGK-1 expression was blocked by JNK inhibitor SP600125, but moderately by MEK1 or p38 inhibitors (Figure 2H).
Figure 2. MAPK pathway mediates stretch-induced SGK-1 expression in venous SMCs. A, Effect of stretch on MAPK phosphorylation. SMCs were exposed to stretch after pretreatment with or without MAPK inhibitors U0126 (5 μmol/L), SB203580 (10 μmol/L), and SP600125 (10 μmol/L). The phosphorylated ERK1/2, p38, and pJNK were detected by Western blot. B, Effect of inhibitors of MAPK signaling pathway on SGK-1 expression. The MAPK inhibitors were applied as in A, and the SGK-1 protein level was detected after mechanical stretch for 4 hours. C and D, Effect of dominant-negative (DN) forms of MAPK on SGK-1 protein level (C) and transcriptional activity (D). E and F, MEK1 but not PI3K or Egr-1 mediated SGK-1 expression in venous SMCs. SMCs were treated with mechanical stretch after pretreatment with U0126 or Ly294002, and SGK-1 mRNA (E) and protein (F) levels were detected by RT-PCR and Western blot. Venous SMCs isolated from Egr-1 knockout mice was also used to detect SGK-1 expression after mechanical stretch (F). G, SGK-1 expression was induced in wire-injured artery. Wire injury was applied to mice common carotid artery, and the injured vessel was collected after 3 hours and immunofluorescence-stained with SGK-1 (green) and α-SMA (red). H, SGK-1 expression was stimulated by growth factors. SMCs were pretreated with MAPK inhibitors for 30 minutes, followed by exposure to IGF-1 (10 ng/mL), PDGF-BB (10 ng/mL), and angiotensin II (1 μmol/L) for 4 hours; the cells lysates were prepared, and SGK-1 protein level was detected by Western blot. The data represent 3 repeated experiments.
Knockout of SGK-1 Blocks Mechanical Stretch–Induced Proliferation in Venous SMCs

To examine whether SGK-1 is necessary and sufficient for mechanical stretch–induced proliferation of venous SMC, we isolated venous SMCs from SGK-1 knockout mice and infected with recombinant adenovirus containing SGK-1, there was a virus dose-dependent SGK-1 expression in venous SMCs (Figure 3A). As expected, stretch increased wild-type venous SMC number and bromodeoxyuridine (BrdUrd) incorporation (Figure 3B and 3C); however, knockout of SGK-1 suppressed the stretch-induced venous SMC proliferation by 83.5% (BrdUrd incorporation experiments, \( P < 0.01 \)) (Figure 3C). Note that overexpression of SGK-1 in unstretched wild-type venous SMCs did not increase proliferation; this suggests that the SGK-1 function not only depends on its expression but also on its activation. When venous SMCs from SGK-1 knockout mice were infected with SGK-1 expressing adenovirus, the stretch-stimulated cell proliferation was restored (Figure 3B and 3C). Because cyclin D1 expression is known for its important role in growth factor stimulated cell cycle progression,25 we examined whether knockout SGK-1 affects cyclin D1 expression. We found that knockout SGK-1 blunted stretch-induced cyclin D1 expression, which was restored by reexpression of SGK-1 (Figure 3D). These results suggested that SGK-1 promotes cell proliferation in response to mechanical stretch.

Mechanical Stretch–Activated SGK-1 Promotes Cell Cycle Progression by Stimulating Cytoplasmic Translocation of p27\(^{kip1}\)

In cancer cells, activated AKT promotes cell cycle progression by regulating its cell cycle inhibitor p27\(^{kip1}\). To determine how mechanical stretch activates SGK-1, we examined whether mechanical stretch activated SGK-1 and stimulated the cytoplasmic translocation of p27\(^{kip1}\). As shown in Figure 4A, mechanical stretch caused the cytoplasmic translocation of p27\(^{kip1}\) in wild-type SMCs, but this translocation was blunted in SGK-1 knockout SMCs. When SGK-1 was reexpressed in SGK-1 knockout SMCs, mechanical stretch restored the cytoplasmic translocation of p27\(^{kip1}\) (Figure 4B). These results suggested that SGK-1 promotes cell cycle progression by stimulating the cytoplasmic translocation of p27\(^{kip1}\).
mechanical stretch–activated SGK-1 promotes venous SMC proliferation, we examined its effect on regulation of p27\textsuperscript{kip1}.

Using immunofluorescence analysis, p27\textsuperscript{kip1} was mainly located in the nucleus in control cells, and translocated to cytosol after stretch treatment (Figure 4A). However, stretch could not stimulate translocation of p27\textsuperscript{kip1} from nucleus into cytosol in SGK-1 knockout cells. Importantly, this p27\textsuperscript{kip1} cytosol translocation was rescued by reexpressing wild-type SGK-1. To further clarify that SGK-1 is a kinase that mediates p27\textsuperscript{kip1} nuclear exportation, we infected venous SMCs with adenovirus containing a dominant-negative form of SGK-1 (127KM), we found that stretch-induced p27\textsuperscript{kip1} nuclear exportation was blocked by the expression of SGK-1 (127KM) (Figure 4A). Taken together, the stretch induced exportation of p27\textsuperscript{kip1} was dependent on active SGK-1. To determine the relationship between activated SGK-1 with phosphorylation of p27\textsuperscript{kip1}, we found that mechanical stretch induced SGK-1 expression (peaked at 2 hours), and this was accompanied with maximal phosphorylation of p27\textsuperscript{kip1} at 2 hours. Note that increased phosphorylation of AKT was appeared as early as at 30 minutes (Figure 4B). Moreover, the stretch-induced phosphorylation of p27\textsuperscript{kip1} was dramatically attenuated in SGK-1 knockout venous SMCs. Re-expressing SGK-1 completely restored the response of these cells to stretch (Figure 4C). There is no change on phosphorylation of AKT between SGK-1 knockout cells and WT cells.

IGF-1R and mTORC2 Are Upstream Signals for Mechanical Stretch–Induced SGK-1 Activation

It is known that fully activation of SGK-1 kinase needs phosphorylation at both Thr256 and Ser422. SGK-1 is a downstream target of PI3K pathway. We have previously shown that mechanical stretch activates IGF-1R and this event plays an important role in stretch-stimulated cell proliferation.\textsuperscript{11} To determine whether SGK-1 function links to IGF-1R signaling pathway, we isolated venous SMCs from IGF-1R–LoxP mice, using recombinant cre (adenovirus Cre) to knockout IGF-1R (Figure 5A). Stretch treatment induced phosphorylation of SGK-1 (Ser422 and Thr256) in control cells; however, these phosphorylations were significantly reduced in IGF-1R knockout cells (Figure 5B). Furthermore, stretch treatment induced phosphorylation of p27\textsuperscript{kip1} was completely restored in the response of these cells to stretch (Figure 4C). There is no change on phosphorylation of AKT between SGK-1 knockout cells and WT cells.
IGF-1R inhibitor, PPP, suppressed SGK-1 phosphorylation on both Ser422 and Thr256 sites (Figure 5C). To determine downstream signals of IGF-1R that activate SGK-1, we found that mTORC2 inhibitor Ku-0063794 blocked stretch- or IGF-1-induced SGK-1 phosphorylation. However, mTORC1 inhibitor rapamycin inhibited stretch-induced phosphorylation of p70S6K, but failed to inhibit SGK-1 phosphorylation, indicating that mTORC2 is a downstream signal of IGF-1R to activate SGK-1 (Figure 5C). Finally, the stretch-induced cell proliferation (Figure 5D) and BrdUrd incorporation (Figure 5E) were significantly reduced by inhibiting or knockout IGF-1R in venous SMCs.

**Stretch-Induced Cytosol Translocation and Phosphorylation of p27kip1 Are Blocked by Inhibition of PI3K**

To provide additional mechanistic evidences that stretch-activated SGK-1 leads to venous SMC proliferation, we inhibited PI3K and examined stretch-induced cell proliferation. Mechanical stretch increased cell number and 2-fold induction of BrdUrd incorporation (Figure 6A and 6B). This increase in proliferation was completely inhibited by the PI3K inhibitor Ly294002 but not by AKT inhibitor (Figure 6A). Consistent with these results, Western blotting demonstrated that inhibition of PI3K also suppressed phosphorylation of p27kip1 (Ser10) (Figure 6C). Furthermore, immunofluorescence staining showed that the number of α-SMA–labeled cells was largely reduced in the vein grafts in SGK-1 knockout mice (Figure 7B). Vein grafts collected from SGK-1 knockout mice had a significant higher lumen/neointima ratio (bigger lumen area with less neointima area) compared with that of graft from wild-type mice (Figure 7B). Vein grafts collected from SGK-1 knockout mice had a significant higher lumen/neointima ratio (bigger lumen area with less neointima area) compared with that of graft from wild-type mice (Figure 7B).

Knockout of SGK-1 Suppresses the Venous SMC Proliferation in Vein Graft

To obtain in vivo evidence, vena cava veins from wild-type or SGK-1 knockout mice were placed into carotid artery of wild-type or SGK-1 knockout mice, respectively. The vein grafts collected at 4 weeks were examined for morphology. Hematoxylin/eosin staining indicated that vascular wall was much thinner in vein grafts from SGK-1 knockout mice compared with that of graft from wild-type mice (Figure 7A). Immunostaining with the smooth muscle cell marker, α-SMA showed that the number of α-SMA–labeled cells was largely reduced in the vein grafts in SGK-1 knockout mice (Figure 7B). Vein grafts from SGK-1 knockout mice had a significant higher lumen/neointima ratio (bigger lumen area with less neointima area) compared with that of graft from wild-type mice (Figure 7B). Vein grafts from SGK-1 knockout mice had a significant higher lumen/neointima ratio (bigger lumen area with less neointima area) compared with that of graft from wild-type mice (Figure 7B). Moreover, there were ~80% of p27kip1 located in the nucleus and <10% in cytosol in smooth muscle cells in normal vein, in which most of the cells are quiescent. The cytosol-localized p27kip1 increased to 42.0±9.6% in neointima smooth muscle cells in wild-type vein grafts, indicating a proliferation of smooth muscle cells, with only 11.1±1.7% cells with nuclear p27kip1. But this pattern of p27kip1 localization was reversed in vein graft created in SGK-1 knockout mice, which most of p27kip1 were remained in nucleus (Figure 7E and 7F). Finally, the proliferation status was determined in these vein grafts. Approximately 62.0±12.2% of PCNA-positive cells were detected in wild-type vein graft; this number decreased to 12.1±3.6% of PCNA-positive cells in vein graft from SGK-1 knockout mice (Figure 7G and 7H). These results indicated that knockout of SGK-1 inhibits cell proliferation and p27kip1 cytosol translocation in vein grafts.
Discussion

The major findings of our study include that mechanical stretch stimulates the transcription and kinase activation of SGK-1 in venous SMCs. MEK1 signaling pathway mediates mechanical stretch–induced SGK-1 expression, and that the IGF-1R/mTORC2 is responsible for mechanical stretch–induced SGK-1 phosphorylation and activation. Our results provided experimental evidence that SGK-1 but not AKT plays an important role in the mechanical stretch–induced proliferation in venous SMCs. Mechanical stretch–activated SGK-1 is responsible for phosphorylation and cytoplasmic translocation of p27\(^{kip1}\) through the activation of IGF-1R/PI3K/mTORC2. Finally, our in vivo experiment revealed that knockout of SGK-1 suppresses the venous SMC proliferation and neointima formation in vein grafts.

Neointima formation in vein graft is the result of excessive vascular cell (including venous SMC) proliferation in which mechanical stretch plays an important role. Our present study identified a cellular signaling mechanism by which stretch induces proliferation of venous SMCs. First, our results demonstrated that SGK-1 is a kinase: its expression is regulated by stretch (Figure 1). In addition, we found that growth factors or wire injury of artery could also increase the expression of SGK-1 (Figure 2G and 2H), suggesting that elevated expression of SGK-1 could involved in the general process of neointima formation. Moreover, we found that the signaling pathway for mechanical stretch–induced SGK-1 expression is mediated by MEK1/ERKs but not other types of MAPK (p38 or JNK) or PI3K or transcriptional factor Egr-1 (Figure 2A through 2F).
Importantly, we found that the kinase activity of SGK-1 is induced by stretch and this response, but not activated AKT, mediates stretch-induced venous SMCs proliferation (Figure 6B and 6C). Growth factor has been reported to stimulate the proliferation of cells by promoting G1 to S phase progression and cyclin D1 induction via PI3K-dependent fashion.26 Our result showed that activated SGK-1 is involved in cyclin D1 expression because knockout of SGK-1 is associated with reduction of the cyclin D1, whereas reexpression of SGK-1 rescued the effect of stretch on cyclin D1 expression (Figure 3D).

In our previous publication, we showed that stretch not only increases the transcription of IGF-1 and its receptor, but also activates IGF-1R and its downstream kinase PI3K signaling pathway.27 Our present study showed that inhibition of IGF-1R and mTORC2, but not mTORC1, prevented stretch-induced SGK-1 activation and phosphorylation of p27kip1 (Figures 4 and 5). Our results are consistent with a recent report of that mTORC2 phosphorylate the C-terminal hydrophobic motif of SGK-1 at Ser422. This creates a docking site for PDK1 to phosphorylate a threonine residue located at the activation loop Thr 256.14,28

Second, we demonstrated that stretch-activated SGK-1 caused proliferation by regulating phosphorylation and cytoplasmic translocation of cell cycle inhibitor p27kip1 (Figure 4A). Phosphorylation at serine 10 and threonine 187 of p27kip1 leads to its exit from the nucleus and its degradation by the proteasome in the cytoplasm, respectively.27 Our data showed that p27kip1 phosphorylation is increased in the cytoplasm of venous SMC subjected to stretch (Figure 4A and 4B and Figure 5B). These data provided experimental evidence that stretch-activated SGK-1 promotes cell cycle via regulation of p27kip1. Our results are consistent with reports of that activated SGK-1 kinase can phosphorylate p27kip1.20

It is generally believed that AKT is a critical signaling kinase for downstream of PI3K pathway in survival and proliferation of vascular cells.30–32 However, our results clearly indicate that SGK-1 rather than its “sister” AKT is the mediator of mechanical signaling events. We found that stretch-induced activation of SGK-1 more closely parallels the increase in p27kip1 phosphorylation than activation of AKT. Although both SGK-1 and AKT can phosphorylate p27kip1, SGK-1 and AKT responds differently to mechanical stretch. Using an inhibitor for AKT, we found that stretch-induced phosphorylation and downregulation of p27kip1 was unaffected. Furthermore, inhibiting AKT did not affect the translocation of p27kip1 from the nucleus. However, phosphorylation and nuclear exportation of the p27kip1 were blocked by a selective PI3K inhibitor (Figure 6D), suggesting that other kinase other than AKT regulates p27kip1. We showed on the other hand, knockout of SGK-1 or dominant-negative SGK-1 (127KM) inhibited stretch-induced phosphorylation and nuclear deporting and degradation of p27kip1. These data provided experimental evidence that SGK-1 but not AKT regulated phosphorylation and exportation of p27kip1 in response to mechanical stretch. This conclusion is supported by our results linking a functional role of SGK-1 to stretch-induced proliferation. Knockout of SGK-1 inhibited stretch-induced proliferation in vitro (Figure 3) and in mouse vein graft model (Figure 7). These data demonstrate an important role of SGK-1 in the stretch-induced proliferation of venous SMC.

To better indicate the interrelationship, Figure 8 depicts the signaling pathway linking mechanical stretch, IGF-1R, and SGK-1 phosphorylation. In summary, our results provide experimental evidence that SGK-1 is a mechanosensitive kinase that leads to activation of venous SMCs. Mechanical stretch induced SGK-1 expression through MEK1/ERK1/2 signaling pathway; in the meantime, mechanical stretch also activated IGF-1R signaling pathway, to phosphorylate SGK-1 at Ser422 and Thr256 sites. mTORC2 was found to be involved in IGF-1R–mediated SGK-1 phosphorylation. Both sites of phosphorylation led to full activation of SGK-1, which then caused p27kip1 nuclear exportation and cell proliferation (Figure 8). Our study demonstrates that mechanical stretch–activated SGK-1 could be an essential intracellular signal, leading to accumulation of vascular cells in vein graft, contributing to neointima formation and possible failure of vein grafts.

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Disclosures
None.

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Novelty and Significance

What Is Known?

- Vein graft failure is commonly associated with neointima formation.
- Mechanical stretch induces vascular smooth muscle cell (VSMCs) proliferation.
- Expression and activation of growth signals are involved in neointimal formation.

What New Information Does This Article Contribute?

- SGK-1 (serum- and glucocorticoid-regulated kinase-1) is a mechanosen-
sitive, growth-promoting kinase. Mechanical stretch stimulates the expression and activation of SGK-1 in VSMCs.
- Knockout of SGK-1 suppresses neointima formation and preserves vein graft patency in mice.

Mechanosensitive, growth-promoting signals involved in the proliferation of VSMCs have not been identified. We found that SGK-1 mediates the proliferation of VSMCs in a model of a vein graft in mice. The mechanism which stimulates SGK-1 mRNA expression involves mechanical stretch–induced activation of the MAPK pathway. Stretch activates both IGF-1R/PI3K and mTOR complex 2, and these 2 responses phosphorylate SGK-1 on threonine 256 and serine 422, respectively, leading to activation of SGK-1 kinase. The consequence of increased SGK-1 expression and activation include exportation of p27kip1 from the nucleus, leading to cell cycle activation and VSMC proliferation. Our results show that in mice lacking SGK-1, the nuclear content of p27kip1 is increased, VSMC proliferation is decreased, and neointima formation is suppressed. These findings identify SGK-1 as a key mediator linking mechanical stretch to neointima formation. Inhibitors of SGK-1 may be useful in preventing neointima formation and vein graft failure.
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The authors regret this error. This error has been noted and corrected in the online version of the article, which is available at http://circres.ahajournals.org/cgi/content/full/107/10/1265

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SUPPLEMENT MATERIAL

Material and methods:

Reagents and virus. Penicillin, streptomycin, DMEM, and FBS were obtained from Invitrogen Life Technologies (Carlsbad, CA). The protein assay kit was purchased from Bio-Rad (Hercules, CA). The antibodies against SGK-1, pAKT (Ser473), pERK1/2, pp38, ppJNK, MEK1, IGF-1R, cyclin D1 and the mTORC1 inhibitor Rapamycin, MEK inhibitor U0126, and substrate GST-GSK3 were purchased from Cell Signaling Technology (Beverly, MA); PCNA and p27kip1 were purchased from Abcam (Cambridge, MA), and pSGK-1 (Thr256) were purchased from Upstate Biotechnology (Billerica, MA); p-ribosome S6K antibodies (pp70S6K-Thr421/Ser424) was purchased from New England Biolabs (Ipswich, MA); Antibodies to total AKT, total JNK, pSGK-1 (Ser422), GAPDH, the secondary antibodies, horseradish peroxidase–linked anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IGF-1R inhibitor picropidophyllin (PPP), PI3K inhibitor LY294002, AKT inhibitor II, MAPK p38 inhibitor SB203580 and JNK inhibitor SP600125, were obtained from Calbiochem (Gibbstown, NJ). Anti-flag affinity agarose gel, antibodies against Flag-M2 and SMA-α, growth factor IGF-1 and angiotensin II were purchased from Sigma, Inc (Sigma-Aldrich, Louis, MO); the mTORC2 inhibitor, Ku-0063794 was from Selleck Chemicals LLC (Houston, TX).

Adenoviruses for expressing SGK-1 or SGK-1 127KM were constructed by cloning human SGK-1 cDNA into pAdTrack-CMV plasmid (Clontech, San Jose, CA) using the AdEasy system1. JNK APF dominant negative (DN) vector2 and flag-MKK6 DN
(K82A)³ was purchased from Addgene (Cambridge, MA). MEK1 DN expression adenovirus was as described⁴.

**Mice and Vein Graft Procedure.** Mouse protocols were approved by Institutional Animal Care and Use and Committee. Wild type B6/129S mice aged 3 month were purchased from Jackson Laboratory. SGK-1 knockout mice were described elsewhere⁵, ⁶. IGF-1R-LoxP mice were obtained by inbreeding as described⁷. The vein graft procedure was similar to that we described earlier⁷.

**Mice wire injury model.** Male 8-week-old B6/129S mice were anesthetized with sodium pentobarbital. The left common carotid artery was injured with a flexible guidewire inserted from the external carotid artery. The guidewire passed the carotid arteries 3 times to make sure the endothelial denudation. Carotid arteries were excised 3 hours after wire injury and perfused with 4% paraformaldehyde, then were collected and snap-frozen in liquid nitrogen.

**Immunohistochemistry.** For vein graft histological analysis, perfusion was performed as described previously¹¹.

**Immunofluorescence staining.** Briefly, cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized in PBS containing 0.1% Triton X-100 and blocked by incubating in 5% bovine serum albumin for 30 min. Fixed cells were washed with PBS and incubated for 60 min at room temperature or overnight at 4 °C with SGK-1 primary antibodies. After washing 3 times, the cells were stained with Fluor secondary antibodies. Images were obtained using a deconvolution microscope with a Zeiss Plan Apochromat 63 ×, 1.4 N.A. objective lens.
Cell Culture. Mouse venous smooth muscle cell were isolated from the inferior vena cava vein of wild type mice, SGK-1 knockout mice or IGF-1R-LoxP mice and cultured as monolayers in DMEM supplemented with 10% heat-inactivated fetal bovine serum. The purity of cells was determined by positive immunostaining cells with SMA-α antibody.

Plasmids and transfection: pSGK1 promoter plasmid was provided by Dr. Richards (Baylor College of Medicine, Houston, TX). Venous SMCs were transfected with plasmids using Nucleofector (Lonza Walkerrsville Inc., Walkersville, MD) according to the manufacturer's protocol.

SGK1 kinase assay. Flag-SGK1 was immunopurified with Flag-M2 beads from venous SMCs transfected with pcDNA3.1-flag-SGK-1 and incubated with 1 µg of GST-GSK3 crosstide (CGPKGPGRRGRRTSSFAEG) in a volume of 30 µl kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2, and 100 µM ATP and 8 µCi [γ-32P]ATP) for 10 min at 30°C. Kinase reactions were performed8. The reactions were stopped by adding SDS sample buffer and heating for 5 min at 95°C. Twenty microliters of the sample was loaded on 10% SDS-PAGE gels, separated and the level of GSK3 phosphorylation was determined by autoradiography. Part of the reaction samples was used for Western blot to asses SGK1 level and GAPDH.

Mechanical stretch and inhibition of intracellular signals. Venous SMC were plated on silicone elastomer-bottomed and collagen-coated plates (Flexcell, McKeesport, PA). Cyclic deformation (60 cycles/min) and 15% elongation of elastomer-bottomed plates were used as described7. In selected plates described below, 5 µM AKT inhibitor, or 10 µM Ly294002, or 10 µM PPP, 5 µM U0126, 10 µM SB203580, 20 µM SP600125, 100
nM Rapamycin, 10 µM Ku-0063794, 10 µg/ml actinomycin D were added to each well 30 min before the start of the stretch-relaxation process.

Real-time PCR. Total RNA from control vena cava vein and vein grafts was isolated using the RNeasy kit (Qiagen, Valencia, CA). The cDNA was synthesized, and real-time PCR was run with the Opticon real-time PCR machine (MJ Research, Waltham, MA). The specificity of real-time PCR was confirmed via routine agarose gel electrophoresis and melting-curve analysis. The housekeeping gene GAPDH was used as an internal standard. The primers used in this study were as follows: mouse SGK-1: forward 5’-TTCGTTAGCCTTTGGTGGAGTTGC-3’; reverse 5’-AGCACCACGTTGGAAGGAAGAGAA-3’; and GAPDH forward 5’-AG TGGGAGTTGCTGTTGAAATC-3’, reverse 5’-TGCTGAGTATGTCGTGGAGTCTA-3’.

BrdU Incorporation. BrdU immunostaining was performed according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN).

Western Blot Analysis. Cells were lysed in RIPA buffer, and 30 µg of proteins were separated by electrophoresis on SDS-polyacrylamide gels and transferred onto PVDF membranes. Membranes were incubated with specific antibodies as described7.

References:


