Akt Controls Vascular Smooth Muscle Cell Proliferation In Vitro and In Vivo by Delaying G1/S Exit

Eugenio Stabile, Yi Fu Zhou, Motoyasu Saji, Marco Castagna, Matie Shou, Timothy D. Kinnaird, Richard Baffour, Matthew D. Ringel, Stephen E. Epstein, Shmuel Fuchs

Abstract—Constitutive activation of serine/threonine kinase Akt causes uncontrolled cell-cycle progression in different cell types and in malignancy. To investigate how Akt activation modulates cell-cycle progression in vascular smooth muscle cells (SMCs) in vitro and in the intact animal, we inhibited Akt-dependent signaling by adenovirus-mediated transfection of a dominant-negative Akt mutant (AA-Akt). We observed reduced proliferation rate ($P<0.01$), DNA synthesis ($P<0.01$), and a significant arrest in G1/S exit ($P<0.01$) both in vitro in response to serum stimulation and in vivo after vascular injury. In vivo transfection of the balloon-injured vessel with AA-Akt reduced SMC proliferation, resulting in decreased neointima compared with control virus ($P<0.01$). These effects were at least in part modulated, both in vitro and in vivo, by increased p21$^{CIP1}$ expression, as demonstrated by lack of effect of AA-Akt on cell proliferation in p21$^{-/-}$ mouse SMCs. In conclusion, this study demonstrates that Akt-dependent signaling enhances cell-cycle progression of nontransformed SMCs in vitro and in response to vascular injury in the intact animal. These results suggest a role for Akt signaling in modulating the response of normal tissues to stress and the response of the arterial wall to acute and possibly repetitive injuries that ultimately contribute to restenosis and atherosclerosis. (Circ Res. 2003;93:1059-1065.)

Key Words: Akt ▪ p21 ▪ restenosis ▪ cell cycle ▪ smooth muscle cell

Cellular processes involved in atherogenesis and restenosis critically depend on proliferative signaling pathways of vascular smooth muscle cells (SMCs) responding to vessel injury.1 Knowledge of the molecular modulators of such pathways would provide insight not only into how SMCs respond to stress but also into potential mechanisms contributing to such vasculoproliferative diseases.

In this regard, Akt defines a family of closely related, highly preserved serine/threonine kinases that have multiple downstream targets able to control cell-cycle progression and apoptosis.2 Moreover, when Akt regulation is lost so that it is constitutively active, cell proliferation is increased and apoptosis inhibited, leading to malignancy progression.2,4 Excessive accumulation of SMCs is a hallmark of both atherosclerosis and restenosis.5 However, it is not known whether Akt-mediated proliferative effects are relevant to the proliferation of SMCs during the arterial wall response to injury and whether this pathway is a potential therapeutic target. Accordingly, in the present investigation, we tested the role of Akt inhibition on injury-induced SMC proliferation and neointima development.6

Materials and Methods

Cell Culture, Vectors, and Gene Transfer

Primary rat aortic, mouse wild-type, and mouse p21$^{-/-}$ (Jackson Laboratories, Bar Harbor, Maine) aortic SMCs were isolated by explantation, cultured, and used before the seventh passage. Cell growth was determined by hemocytometer.

Replication-defective adenovirus expressing bacterial β-galactosidase (β-gal) and dominant-negative Akt mutant (AA-Akt) under the control of the CAG eukaryotic promoter were used in the study.7,8 Distal to the Akt mutant gene, the adenovirus expressing AA-Akt encodes for HA epitope used for tagging and confirmation of the transgene expression.

A multiplicity of infection of 20 and 50 provided an infection efficiency >95%, respectively, in rat and mouse SMCs. Cells were subjected to experiments 48 hours after infection.

[1H]Thymidine Incorporation

Cells were plated in 96-well plates, and 48 hours after infection, 1 μCi of [1H]thymidine was added to each well and incubated overnight in 10% FBS. Average [1H]thymidine incorporation was assessed as described9 and expressed as counts per well. Average counts per cell (counts/cell) was determined by dividing average counts per well by average number of cells counted in 8 wells per group after [1H]thymidine incubation.

Cell-Cycle Analysis

Primary SMCs were infected with adenoviral vectors and synchronized in G1/G0, by serum deprivation for 48 hours and then stimulated by 10% FBS to enter the cell cycle.10,11 DNA content was assessed using a flow cytometric DNA analysis.12

Preparation of Cellular Lysates and Western Immunoblot Analysis

Cells were lysed in ice-cold buffer,13 and lysates were collected. Five micrograms of protein was separated on denaturing SDS-PAGE 10%
and then blotted onto nitrocellulose (Invitrogen) by wet electroblotting. Blots were blocked overnight at 4°C with 5% nonfat dry milk in 0.1% TBS-T and then incubated with the primary antibody (dilution 1:1000 for anti-proliferating cell nuclear antigen [PCNA], p21, Ser 411 p-p70S6K, HA, p27, cyclin A, cyclin E, cyclin D3; Santa Cruz Biotechnology, Santa Cruz, Calif) (dilution 1:1000 for Akt and Ser 473 p-Akt; Cell Signaling). Specific proteins were detected by enhanced chemiluminescence according to the manufacturer’s instructions (Pierce).

**Akt Kinase Assay**

Akt kinase activities were measured using a nonsotopic Akt kinase assay kit (Cell Signaling). Briefly, after selective immunoprecipitating of Akt from cell lysates with an antibody against Akt, the precipitate was incubated with GSK-3 fusion protein in the presence of ATP and GSK-3β phosphorylation was measured by Western blotting using a Ser 219 p-GSK-3αβ antibody (Cell Signaling).

**Arterial Injury and Adenovirus-Mediated Gene Transfer In Vivo**

The study was approved by the Animal Care and Use Committee of the MedStar Research Institute. Twenty-two adult male Sprague-Dawley rats (350 g; Jackson Laboratories, Bar Harbor, Maine), anesthetized with ketamine (100 mg/kg, IM) and xylazine (10 mg/kg, IM), were subjected to balloon injury of the right common carotid artery. After injury, 0.1 mL of recombinant adenovirus (5 × 10⁹ pfu/mL) was instilled into a 1.5-cm isolated segment of the distal ipsilateral common carotid artery and allowed to dwell for 45 minutes. Intraparenchymal heparin (70 U/kg) was injected to obtain sufficient anticoagulation. Overall efficiency of infection was estimated to be ~35% of the nuclei present in the vessel wall at 2 days after transfection.

**Localization of Recombinant Adenovirus Expression and Cell Proliferation in the Vessel Wall**

Two days after instillation of adenovirus encoding β-gal (n = 3), lacZ gene expression was detected by β-galactosidase staining (Invitrogen). The samples were divided into 2-mm-thick segments, over-laid with OCT compound, and frozen in liquid nitrogen. Cryostat sections (7 μm) were mounted on slides. For cell proliferation assessment and apoptosis detection, arteries were collected 2 days after the experimental procedure and analyzed by immunohistochemistry technique for Mib-1 nuclear expression (Ki-67 antibody, Santa Cruz Biotechnology) or TUNEL.

**Measurements of Arterial Wall Protein Expression and Cell Proliferation**

Expression of α-smooth muscle actin (α-SMA) and collagen type I (Coll I) was assessed immunochemically using antibodies against α-SMA and Coll I and analyzing sections by planimetry. The wall area was considered significant.

**Vascular Ultrasound Imaging**

Vascular ultrasound imaging studies were performed at 14 days (before euthanasia), after topical administration of lidocaine. The studies were performed with a commercially available imaging system (Boston Scientific/SciMed Corporation), which incorporated a single-element 30- or 40-MHz beveled transducer mounted on the tip of a flexible shaft and rotated at 1800 rpm within short monorail imaging sheath. After surgical exposure of the carotid artery, a catheter was placed parallel to the vessel, with the tip located at the bifurcation of the right common carotid and the innominate arteries. To reduce any impact on the vasomotor tone of the artery, both transducer and artery were covered with a 2% lidocaine solution. After placement, the transducer was automatically withdrawn retrograde to a point corresponding to the carotid bifurcation using motorized pullback at 0.5 mm/sec. Quantitative analysis included minimal lumen cross sectional area (CSA).

**Morphology**

Fourteen days after injury and viral infection, rats (n = 16) were killed and carotid vessels were perfusion-fixed with 4% (wt/vol) formaldehyde and harvested for paraffin embedding. Sections (5 μm) were stained with H&E. The neointima area (NI) and medial area (M) of the most stenotic cross section were measured by digital planimetry, and the NI/M ratio was calculated.

**Statistical Analysis**

All results are presented as mean ± SEM. Unpaired Student’s t test was used to compare nominal and normalized values. P value of 0.05 was considered significant.

**Results**

We infected cultured SMCs with a recombinant adenovirus encoding a phosphorylation-resistant Akt mutant (Thr308→ALA and Ser473→ALA). Phosphorylation of Thr and Ser is necessary for Akt activation and for achievement of maximal Akt activity, respectively. This particular Akt mutant, when overexpressed in cells, acts in a dominant-negative manner, thereby inhibiting Akt signaling.

We first confirmed serum-induced Akt activation of primary SMCs in our system, an effect that was not substantially altered by infection with our control adenovirus encoding β-gal (Figure 1A, top). In the same system, at 48 hours after infection with an adenoviral vector, AA-Akt was efficiently expressed (Figure 1A, middle). Biological activity of the dominant-negative Akt was evidenced by its ability to reduce Akt kinase activity (Figure 1A, bottom) as well as the phosphorylation of p70S6K (Figure 1A, middle), a downstream target of Akt, which, when activated, reduces G1 phase duration.

Over 96 hours, AA-Akt transfection of SMCs reduced cell proliferation in vitro (Figure 1B). This was associated with reduced DNA synthesis at 48 hours after infection, assessed by [³H]thymidine incorporation (Figure 1C, top). To control for possible confounding effect of reduced number of viable cells in the AA-Akt–transfected cells (attributable to apoptosis triggered by Akt inhibition), the number of counts per well was normalized by dividing it by the average number of cells in each well, counted after the [³H]thymidine incubation. Normalized counts (counts/cell) were consistently reduced by 50% (Figure 1C, bottom). The decrease in SMC DNA synthesis was attributable to delayed G1/S exit, because we observed a significant reduction in the number of cells entering S-phase 24 hours after induction with 10% FBS in the AA-Akt–transfected cells compared with controls (Figure 2A). Importantly, infection of cells with the adenovirus containing the reporter transgene did not alter the percentage of cells entering S-phase.

These findings were supported by a consistent increase in p21Cip1 expression in the AA-Akt–transfected cells that reached its maximum 24 hours after serum induction (Figure 2B). That is the time of G1-S transition in our experimental model. Quantitative analysis of p21Cip1 expression 24 hours after serum stimulation showed that Ad.AA-Akt infection increases p21 expression more than 2-fold compared with control (+P < 0.01) (data not shown). At the same time point, we did not observe any changes in p70S6K or other cell-cycle
As confirmatory evidence of reduced cell proliferation, reduced expression of PCNA was observed after Ad.AA-Akt infection. To better understand the role of p21 Cip1 in inhibition of SMC proliferation after blocking Akt-dependent signaling, we assessed cell growth in p21Cip1-deficient SMCs after infection with Ad.AA-Akt. The results confirmed that blocking Akt significantly decreases SMC proliferation and that this effect is abolished when p21 Cip1 is eliminated from the cell-cycle regulatory machine (Figure 2D).

On the basis of these in vitro data, we then assessed the role of Akt-dependent signaling in SMC proliferation induced by vascular injury. After having confirmed consistent Akt activation in response to balloon-induced arterial injury in our system (Figure 3A), we evaluated the role of Akt-dependent signaling in vivo using recombinant adenoviruses. Two days after vessel injury and gene transfection (10^8 pfu/mL), we observed control gene (β-gal) expression in 35% of medial SMCs (Figure 3B). Similar to our in vitro studies, 2 days after arterial injury and AA-Akt gene transfer, p21 Cip1 expression in the arterial wall was increased compared with baseline arterial expression and control gene transfer (Figure 3B). This effect was clearly reduced when a log less virus (10^7 pfu/mL) was used to transfect the arterial wall (Figure 3B). Of note, we observed an increase of p-p21 that was abolished by AA-Akt transfection (Figure 3B). At the same time point, we observed a reduction of overall PCNA expression in the arterial wall of AA-Akt–transfected SMCs.
animals and a reduced percentage of Ki67-positive nuclei in the arterial wall (Figures 3D and 3E). In contrast, AA-Akt did not additionally increase the amount of tunnel-positive nuclei already present in the arterial wall after balloon-induced injury (b-gal, 9.5\% ± 3.6\%; AA-Akt, 10.8\% ± 1.01\%; P = NS) Few TUNEL-positive nuclei were detected in the uninjured control vessels (1\% ± 0.57\%).

This result demonstrates that, at least in this system, blocking Akt-dependent signaling does not additionally augment the already large increase in apoptosis induced in the vessel wall after mechanical injury. We believe the most likely explanation for this finding is that the large increase in apoptosis induced by vascular injury per se masked any additional increase in apoptosis that may have been induced by AA-Akt. In total, these data indicate that Akt inhibition abrogates the stimulatory effect of vascular injury on DNA synthesis and cell-cycle progression, ultimately resulting in inhibition of SMC proliferation, and that this mechanism seems to be the dominant one leading decreased neointimal development.

To assess whether this inhibition is also of biologic relevance, we studied neointima formation at 14 days after arterial wall injury and gene transfection. Infection with the vector expressing the AA-Akt transgene (versus infection with the vector expressing β-gal) reduced NI (0.06\% ± 0.01 versus 0.11\% ± 0.008 mm², P < 0.01) and NI normalized to the M (NI/M) (0.45\% ± 0.06 versus 0.79\% ± 0.06, P < 0.01) (Figures 4A and 4B). The reduction in neointima formation was associated with larger minimal lumen CSA, as assessed in vivo by perivascular ultrasound (0.74\% ± 0.04 versus 0.59\% ± 0.03 mm²; P < 0.05) (Figures 4A and 4B).

Discussion

We have demonstrated that in SMCs, inhibition of Akt activation abrogates the stimulatory effect of serum induction...
in vitro and vascular injury in vivo on DNA synthesis, cell-cycle progression, and, ultimately, cell proliferation. The results also suggest that increased levels of p21 Cip1 mediate these effects, both in vitro and in vivo.

The inhibition of the injury-induced proliferation of SMCs, achieved through gene transfer of a dominant-negative mutant Akt to the arterial wall, confirmed the observations of our laboratory and other laboratories of a role of Akt in regulating SMC cell cycling.\(^{10,31,32}\) To test whether this hypothesis is also valid in the in vivo situation, we used an arterial balloon injury model; in the process of balloon injury, the endothelial cell layer is denudated and the vessel wall is subjected to mechanical stretch. We used this model for three important reasons. First, it is traditionally used to model restenosis, because it causes marked SMC proliferation similar to that occurring during the acute injury caused by angioplasty performed in patients with obstructive vascular disease; second, proliferation of medial SMCs is also the hallmark of atherosclerosis, a disease caused in part by the vascular proliferative response to chronic vascular injury, such as induced by various risk factors for atherosclerosis (i.e., hypertension and hyperlipidemia); third, the injured vessel also allows for highly efficient transfection of the SMCs resident in the vessel media when viral vectors are used.\(^{18}\)

Our findings demonstrate a clear role for blocking the Akt-dependent signaling to prevent SMC proliferation after arterial injury. Moreover, our results indicate that Akt controls SMC proliferation, at least in part, by regulating p21 cellular levels. Conflicting data have been reported on how the Akt-mediated signaling regulates p21 levels and activity in many cell types\(^{33-36}\); however, no data are actually available in vivo for SMCs.

In our study, compared with a control uninjured artery, a significant increase in p21 phosphorylation was detected 48 hours after vascular injury. It has been demonstrated in vitro that Akt can associate with p21 and phosphorylate a consensual residue in the nuclear localization signal of p21, leading to the cytoplasmic localization and suppression of its growth-inhibiting activity.\(^{37}\)

Because p21 is a phosphoprotein targeted for posttranscriptional modification by various kinases, phosphorylation of p21 may regulate the interaction of p21 with its binding partners, an effect that could alter p21 stability. In particular, it has been demonstrated, at least in endothelial cells, that p21 levels are dependent on a phosphorylation-dependent degradation.\(^{38}\) In our model of vascular injury, when Akt-dependent signaling is blocked by AA-Akt transfection, there is less phosphorylated p21 and increased total p21 levels.
This suggests that blocking Akt-dependent signaling prevents injury-induced p21 phosphorylation. In the nonphosphorylated state, p21 cannot be recognized by the proteasome and its degradation is thereby inhibited. The resulting intracellular accumulation of p21 leads to cell-cycle arrest.

Our results support a role for Akt in controlling p21\(^{Cip1}\) levels in SMCs and suggest this as a possible mechanism through which Akt regulates cell-cycle progression. Moreover, we showed that p21 is still expressed after SMC proliferation is induced by a mitogenic stimulus (such as mechanical injury to the vessel wall), presumably serving as an important mediator in the negative-feedback loop. A similar finding has been observed after femoral artery injury in a pig model of experimental angioplasty.\(^{39}\) In our system, p21 expression is increased by blocking Akt-dependent signaling, suggesting a role for Akt in determining the timing of this feedback activation.

Our results support a role for Akt in controlling p21\(^{Cip1}\) levels in SMCs and suggest this as a fundamental mechanism through which Akt regulates cell-cycle progression. The demonstration that blocking Akt-dependent signaling in SMCs derived from p21 knockout mice resulted in no inhibitory effect on SMC proliferation establishes that p21 is necessary for the SMC-related effects of Akt. However, extensive and detailed studies will be required to better understand how Akt controls p21\(^{Cip1}\) levels and activity after arterial injury.

Of note, we did not observe any variation in the level of p27\(^{Kip1}\) expression after blocking Akt-dependent signaling, despite the fact that several studies have reported a causal relationship between Akt activation and p27 activity in different cell types. Recently, it has been reported that in vascular SMCs, Akt regulates p27\(^{Kip1}\) levels only under specific conditions. Thus, it was shown that serum-induced protein degradation of p27\(^{Kip1}\) is independent of phosphatidylinositol 3 kinase/Akt signaling; however, mechanical strain (in the absence of growth factors) did repress transcription of p27\(^{Kip1}\). This repression was attributable to inhibition of AFX-like forkhead transcription factor activity.\(^{40}\) AFX-like forkhead transcription factor activity, as in many other cell types, is inactivated by Akt.\(^{41}\)

Although we can speculate that in this animal model a major role could be played by the endothelial-denuded arterial wall, which would expose SMCs to several mitogenic factors, we cannot definitively explain why p27\(^{Kip1}\) levels were not affected by blocking Akt activation. Even though it has been suggested that the extent by which Akt activity determines downstream effectors levels is likely affected by cell type and the activity of other cell-cycle control signaling pathways,\(^{2,42}\) we do believe that more extensive studies will be necessary to solve this issue. In summary, our results indicate that Akt importantly influences cell-cycle progression of nontransformed SMCs, both in vitro and in vivo. The inhibitory effect that Akt inactivation exerts on the prolifer-
ative response to vascular injury renders Akt a potential target for therapeutic interventions aimed at inhibiting the SMC proliferation that occurs in such vasculoproliferative diseases as atherosclerosis and restenosis.

Acknowledgments

This study was entirely supported by internal grants from the Cardiovascular Research Institute.

References


Akt Controls Vascular Smooth Muscle Cell Proliferation In Vitro and In Vivo by Delaying G1/S Exit
Eugenio Stabile, Yi Fu Zhou, Motoyasu Saji, Marco Castagna, Matie Shou, Timothy D. Kinnaird, Richard Baffour, Matthew D. Ringel, Stephen E. Epstein and Shmuel Fuchs

*Circ Res.* 2003;93:1059-1065; originally published online November 6, 2003; doi: 10.1161/01.RES.0000105086.31909.1B

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/93/11/1059

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org//subscriptions/