Rapamycin Promotes Vascular Smooth Muscle Cell Differentiation through Insulin Receptor Substrate-1/Phosphatidylinositol 3-Kinase/Akt2 Feedback Signaling*

Kathleen A. Martin1,2, Bethany L. Merenick1,3, Min Ding1, Kristina M. Fetalvero1, Eva M. Rzucidlo1, Courtney D. Kozul1, David J. Brown1, Helen Y. Chiu2, Maureen Shyu3, Bethany L. Drapeau5, Robert J. Wagner1, and Richard J. Powell5

From the 4Division of Vascular Surgery and 5Department of Pharmacology and Toxicology, Dartmouth Medical School, Lebanon, New Hampshire 03756

The phenotypic plasticity of mature vascular smooth muscle cells (VSMCs) facilitates angiogenesis and wound healing, but VSCM dedifferentiation also contributes to vascular pathologies such as intimal hyperplasia. Insulin/insulin-like growth factor 1 (IGF-I) is unique among growth factors in promoting VSMC differentiation via preferential activation of phosphatidylinositol 3-kinase (PI3K) and Akt. We have previously reported that rapamycin promotes VSMC differentiation by inhibiting the mammalian target of rapamycin (mTOR) target S6K1. Here, we show that rapamycin activates Akt and induces contractile protein expression in human VSMC in an insulin-like growth factor 1-dependent manner, by relieving S6K1-dependent negative regulation of insulin receptor substrate-1 (IRS-1). In skeletal muscle and adipocytes, rapamycin relieves mTOR/S6K1-dependent inhibitory phosphorylation of IRS-1, thus preventing IRS-1 degradation and enhancing PI3K activation. We report that this mechanism is functional in VSMCs and crucial for rapamycin-induced differentiation. Rapamycin inhibits S6K1-dependent IRS-1 serine phosphorylation, increases IRS-1 protein levels, and promotes association of tyrosine-phosphorylated IRS-1 with PI3K. A rapamycin-resistant S6K1 mutant prevents rapamycin-induced Akt activation and VSMC differentiation. Notably, we find that rapamycin selectively activates only the Akt2 isoform and that Akt2, but not Akt1, is sufficient to induce contractile protein expression. Akt2 is required for rapamycin-induced VSMC differentiation, whereas Akt1 appears to oppose contractile protein expression. The anti-restenotic effect of rapamycin in patients may be attributable to this unique pattern of PI3K effector regulation wherein anti-differentiation signals from S6K1 are inhibited, but pro-differentiation Akt2 activity is promoted through an IRS-1 feedback signaling mechanism.

Vascular smooth muscle cells (VSMCs) maintain a phenotypic plasticity that is important in physiological processes such as angiogenesis, and in pathological responses, including atherosclerosis, intimal hyperplasia, and restenosis. Mature VSMCs are quiescent and exhibit a differentiated, contractile phenotype. Differentiation status in vitro can be measured by expression of smooth muscle-specific contractile proteins, including calponin, caldesmon, and smooth muscle myosin heavy chain (SM-MHC) (1). In response to injury, or upon in vitro culture, VSMCs re-enter the cell cycle, proliferate, migrate toward attractants, down-regulate expression of contractile proteins, and up-regulate protein synthesis, particularly of the extracellular matrix. This de-differentiated phenotype is referred to as “synthetic” because of this property (1).

VSMC de-differentiation and resultant intimal hyperplasia in response to vessel injury are common problems following vascular interventions such as angioplasty, stent placement, and bypass grafts. Since receiving FDA approval in 2003, the use of the mTOR inhibitor rapamycin on drug-eluting stents has had a profound impact in reducing the incidence of restenosis and the need for repeat revascularizations in coronary lesions (2, 3). However, recent concerns about the risk of late thrombosis with these stents, likely as a result of impaired re-endothelialization (4), reveals the need for a better understanding of the mechanisms of action of rapamycin on VSMCs to develop VSMC-specific therapies. Although it is known that rapamycin inhibits VSMC migration (5) and proliferation (6) in vitro and intimal hyperplasia in vivo, we have recently demonstrated that rapamycin also induces differentiation in cultures of synthetic

3 The abbreviations used are: VSMC, vascular smooth muscle cell; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; S6K1, S6 kinase 1; IGF-I, insulin-like growth factor I; IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol 3-kinase; GSK-3β, glycogen synthase kinase 3β; SM-MHC, smooth muscle myosin heavy chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HA, hemagglutinin; siRNA, small interfering RNA.
phenotype VSMCs (7). This occurs as a coordinated induction of cyclin-dependent kinase inhibitor (p21cip and p27kip) and contractile protein expression, which begins as early as 2 h following rapamycin treatment. The rapamycin-treated cells reduce protein synthesis by 40–60% and assume a contractile morphology. We have shown that this differentiation is mediated, at least in part, by inhibition of the mTOR effector p70 S6K1 (7).

When associated with the proteins raptor and mLST8, mTOR exists in a rapamycin-sensitive complex (mTORC1) that plays a crucial role in regulating cell growth and proliferation in response to cellular environment, including nutrient, energy, and oxygen sufficiency (8, 9). The importance of this pathway in metabolism, diabetes, obesity, and cancer is becoming increasingly appreciated. Notably, in insulin-responsive tissues such as skeletal muscle and adipocytes, mTOR and S6K1 provide negative feedback to the insulin and insulin-like growth factor I (IGF-I) signaling pathways through inhibitory serine phosphorylation of the insulin receptor substrate-1 (IRS-1), which targets IRS-1 for degradation (10). Inhibition of mTOR/S6K1 with rapamycin relieves this repression of IRS-1 and facilitates its activation of phosphatidylinositol 3-kinase (PI3K) and Akt in response to insulin or IGF-I. To date, this feedback-signaling network, which contributes to insulin resistance in type II diabetes (11, 12), has not been documented in vascular smooth muscle.

IGF-I receptor activation of PI3K and Akt is an important mechanism for maintenance of the differentiated smooth muscle contractile phenotype. Primary SMCs maintain a differentiated phenotype when treated with IGF-I but de-differentiate when exposed to other growth factors, including platelet-derived growth factor, fibroblast growth factor, or epidermal growth factor (13). The IGF-I differentiation signal is thought to be mediated by PI3K and Akt, because inhibition of this pathway with PI3K inhibitors or dominant negative Akt promotes dedifferentiation of vascular (14) or gizzard smooth muscle (15). We have shown that constitutively active myristoylated Akt is sufficient to promote VSMC differentiation (16). The unique ability of IGF-I to promote VSMC differentiation, as opposed to proliferation, has been attributed to its preferential activation of the Akt2 isoform when treated with IGF-I but de-differentiate, at least in part, by inhibition of the mTOR effector p70 S6K1 (7).

The role of Akt signaling in VSMCs is not entirely clear, because Akt activation has been suggested to promote VSMC hypertrophy and neointimal hyperplasia post-injury (17). One reason for the discrepancy in results could be due to differences in Akt isoform substrates. Much of the research performed to date has used reagents to modulate expression of Akt1 and antibodies that do not discriminate among the three Akt isoforms. Notably, recent evidence suggests that different Akt isoforms perform distinct physiological functions (18–21).

In this study, we sought to understand the signal transduction events by which rapamycin promotes VSMC differentiation. Based on mTOR/S6K1 feedback regulation of IRS-1 in other cell types, and the role for PI3K/Akt in IGF-I-dependent VSMC differentiation, we tested the hypothesis that rapamycin promotes VSMC differentiation through Akt activation. Here, we provide extensive evidence to support this feedback mechanism in VSMCs and reveal that specific activation of the Akt2 isoform is essential for rapamycin-induced VSMC differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human VSMCs were isolated from vascular surgery patients or organ donors (approved Institutional Review Board protocol), or human coronary artery VSMCs from organ donors were purchased from Cascade Biologics (Portland, OR). Human VSMCs were cultured in M199 medium with 10% FBS, and supplements as described previously (22). Bovine or rat aortic SMCs were isolated and cultured as described before (7). VSMCs at passages 2–6 were used for experiments. Data in each experiment have been confirmed in VSMCs from at least two different preparations. Twenty-four hours prior to drug treatment, cells were transferred to media containing 0% or 2.5% FBS for the duration of the experiment as indicated in the figure legends. VSMCs cultured in 2.5% FBS proliferate slowly and do not spontaneously differentiate (7, 16, 22). VSMCs were treated with vehicle (ethanol for rapamycin) or drug (rapamycin (Calbiochem) or IGF-I (Upstate, Charlotte, NC)) as indicated in the figure legends.

Western Blotting—Whole cell lysates were prepared and quantitated by Bradford assay as previously described (7, 16, 22). Equal amounts of protein per lane were subjected to SDS-PAGE, transferred to a membrane, and immunoblotted using antibodies against SM2-MHC (Seikagaku America, Cape Cod, MA), h-caldesmon, SMα-actin, calponin 1 (Sigma), GAPDH, anti-phosphotyrosine (PY20), β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), Akt1, Akt2, total Akt, phospho-Ser473 Akt, phospho-Ser-9 GSK-3β, total GSK3-β, S6K1, total S6, phospho-S6 (Ser-235/236), IRS-1 phospho-Ser-636, or phospho-Ser302 (Cell Signaling, Boston, MA), or total IRS-1 (Upstate), horseradish peroxidase-conjugated secondary antibodies, and chemiluminescent detection and autoradiography as described (7, 16, 22). Anti-HA antibody was a kind gift of Dr. John Blenis.

Semi-quantitative Reverse Transcription-PCR—Total RNA was isolated as described before (7, 22), and 500 ng was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad). Titrations of the reverse-transcribed CDNA were performed to determine the linear range of the PCR for all primer sets. PCR was performed using primers for SM-MHC, and pyruvate dehydrogenase, and products analyzed by agarose gel electrophoresis as described (22). Primers for Akt2 were 5′-ATGAATGAGGTGTCTTCTACATCAAAGAAGGC and 3′-TGCTTTAGGGCTGTGGCGACC. SM-MHC or Akt PCR products were normalized to levels of pyruvate dehydrogenase product, a housekeeping gene control.

Immunoprecipitation—Cell lysates were prepared as for Western analysis and protein content measured by Bradford assay. Equal amounts of protein were immunoprecipitated with antibody to p85 PI3K (BD Pharmingen, San Diego, CA), Akt1, Akt2, total Akt, phospho-Ser473 Akt, phospho-Ser-9 GSK-3β, total GSK3-β, S6K1, total S6, phospho-S6 (Ser-235/236), IRS-1 phospho-Ser-636, or phospho-Ser302 (Cell Signaling, Boston, MA), or total IRS-1 (Upstate), h-caldesmon, SMα-actin, GAPDH, anti-phosphotyrosine (PY20), β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), Akt1, Akt2, total Akt, phospho-Ser473 Akt, phospho-Ser-9 GSK-3β, total GSK3-β, S6K1, total S6, phospho-S6 (Ser-235/236), IRS-1 phospho-Ser-636, or phospho-Ser302 (Cell Signaling, Boston, MA), or total IRS-1 (Upstate), horseradish peroxidase-conjugated secondary antibodies, and chemiluminescent detection and autoradiography as described (7, 16, 22). Anti-HA antibody was a kind gift of Dr. John Blenis.
Rapamycin Activates Akt2 in VSMC Differentiation

Rapamycin Activates Akt and Promotes VSMC Differentiation in a Serum-dependent Manner—Primary VSMCs cultured on plastic in serum exhibit the dedifferentiated synthetic phenotype, characterized by low expression of contractile protein markers of differentiation. We have previously demonstrated that rapamycin inhibition of mTOR and S6K1 promotes VSMC differentiation as measured by changes in cell morphology, protein synthesis, contractile protein expression at both the protein and mRNA level, and induction of cyclin-dependent kinase inhibitors p21cip1/p27kip1 (7). Because several lines of evidence implicate Akt in VSMC differentiation (13–16), we examined Akt activity in VSMCs treated with rapamycin. In human or bovine VSMCs cultured in 2.5% serum, rapamycin induced Akt activation, as measured by phosphorylation of the critical serine 473, in a time-dependent manner (Fig. 1). Under these conditions, rapamycin also induced expression of protein markers of differentiation, including SM-MHC and h-caldesmon. Inhibition of mTOR signaling was confirmed by the rapid and complete inhibition of phosphorylation of ribosomal S6, an in vivo S6K1 substrate. Akt serine 473 phosphorylation was observed as early as 30 min after rapamycin treatment and increased in intensity with time (Figs. 1 and 2). Akt activity was also confirmed by the increased phosphorylation of the in vivo Akt substrate GSK3β (Fig. 1C). Electrophoretic mobility shift of the mTOR effector S6K1 (Fig. 2A), which correlates with its phosphorylation status and activity (8), also verified the concomitant mTOR pathway inhibition. Notably, rapamycin activation of Akt only occurred in the presence of serum. Akt activation was observed in cells treated with rapamycin in 2.5% serum, but no activation occurred in serum-free media (Figs. 2 and 3), even after 24–48 h of treatment (Fig. 2B).

Given the serum dependence of Akt activation, we next assessed whether rapamycin-induced differentiation similarly required serum. In contrast to the rapamycin induction of differentiation markers that occurred when cells were cultured in 2.5% serum (Fig. 1), rapamycin treatment failed to induce contractile protein expression in serum-free media (Fig. 3A, left panel), suggesting that the ability of rapamycin to activate Akt and to promote differentiation both require a factor present in serum. Given the previous work from Sobue et al. (14) showing that IGF-I-dependent Akt activation can maintain VSMC dif-

**RESULTS**

**Adenoviral Expression of Rapamycin-resistant S6K1—**Infection with adenovirus encoding an epitope-tagged, rapamycin-resistant, constitutively active mutant of S6K1 (ED3E) and green fluorescent protein, or with a control adenovirus encoding green fluorescent protein, was performed as described (7). Briefly, VSMCs were infected overnight, washed, and medium was changed to M199/2.5% FBS prior to drug treatment.

**siRNA—**Transient transfection of small interfering RNA (siRNA) was performed in human VSMCs using a Nucleofector (Amaxa, Gaithersburg, MD). 1 million cells were transfected with 2–2.5 μg of siRNA and cultured for 48 h. Medium was then changed to 2.5% serum, and cells were treated with vehicle or rapamycin and harvested for reverse transcription-PCR analysis or Western blotting as above. siRNA constructs for Akt1 (SMARTpool), Akt2 (four different siGENOME duplexes), and nonsilencing siRNA (siCONTROL) were purchased from Dharmacon (Lafayette, CO).

**Akt Overexpression—**6 μg of plasmid encoding HA-epitope-tagged wild-type Akt1, Akt2, or myristoylated-Akt2 (courtesy of Dr. Jin Q. Cheng), or pcDNA3 vector was transiently transfected into human VSMCs using the Nucleofector method as above.

**Statistical Analysis—**Student’s paired t-tests were performed with StatView software (Abacus Concepts, Berkley CA). Data are expressed as the mean ± S.E. A significance level of 0.05 was assumed for all tests.

![FIGURE 1. Rapamycin induces Akt phosphorylation and contractile protein expression in VSMCs. Human (A and C) or bovine (B) VSMCs were cultured in M199 or Dulbecco’s modified Eagle’s medium, respectively, with 2.5% FBS for 24 h prior to treatment with 20 nM rapamycin over the time course indicated. Cells were harvested for Western blot analysis with antibodies to contractile protein markers of differentiation (SM2-MHC and h-caldesmon), phospho-Ser-473 Akt, total Akt, phospho-Ser-9 GSK3-β, total GSK3-β, phospho-S-6, or GAPDH or β-tubulin (loading controls). V = cells treated with vehicle for 24 h (A and C) or 48 h (B).](image)
Rapamycin Activates Akt2 in VSMC Differentiation

Furthermore, addition of an anti-IGF-I-neutralizing antibody, but not a control IgG, to serum-free media completely blocked the rapamycin-induced Akt activation (Fig. 3B, right side). These experiments revealed that IGF-I is necessary and sufficient for rapamycin-induced Akt activation.

Rapamycin Activates Akt by Relieving mTOR/S6K1-dependent Negative Feedback to IRS-1—The requirement for IGF-I suggested a potential mechanism by which mTOR inhibition activates Akt and promotes VSMC differentiation in VSMCs. In insulin-responsive tissues (skeletal muscle and adipocytes), mTOR activity dampens PI3K signaling by promoting S6K1-mediated inhibitory phosphorylation of IRS-1 (10, 23), an adaptor necessary for insulin/IGF-I-receptor recruitment of the p85 subunit of PI3K. S6K1-dependent serine phosphorylation of IRS-1 (24–26) sterically hinders receptor-mediated phosphorylation of the tyrosine residues that recruit effector proteins but also targets IRS-1 for degradation (10). We tested the hypothesis that rapamycin promotes Akt activation and VSMC differentiation by inhibiting S6K1-mediated negative feedback regulation of IRS-1.

IRS-1 contains many regulatory phosphorylation sites (27), including tyrosines, which recruit effector-signaling proteins when phosphorylated, and serine/threonine residues, which serve inhibitory roles (10). Changes in IRS-1 serine versus tyrosine phosphorylation result in well documented patterns of mobility shifts in SDS-PAGE (10, 23–25). We examined IRS-1 mobility in VSMCs by Western blot analysis and observed that treatment with rapamycin resulted in a shift in IRS-1 migration from a slower mobility form, consistent with serine phosphorylation, to a higher mobility form, consistent with tyrosine phosphorylation (see arrows, Fig. 4A). This enhanced mobility has been shown to correlate with tyrosine phosphorylated IRS-1 (10, 23–25). In addition to the mobility shift, rapamycin decreased phosphorylation at specific serine residues known to be regulated by S6K1 in other tissue types (serines 302 and 636) (24–26) as determined by immunoblotting with phospho-specific antibodies to these sites. A reduction in serine 636 phosphorylation was observed over a time course of rapamycin treatment in Fig. 4 (B and E). The blot shown in Fig. 4B is from the same samples as Fig. 1B. Thus, the diminished IRS-1 serine phosphorylation correlates temporally with Akt activation and contractile protein induction. The change in IRS-1 mobility and phosphorylation also correlated with an accumulation of IRS-1 protein, consistent with inhibition of IRS-1 degradation. This was apparent after 4 (Figs. 4A and 5A) or 24–48 h of rapamycin treatment (Fig. 4, C and E).

To verify that the rapamycin-induced mobility shift in IRS-1 correlates with functional activation, we
Rapamycin Activates Akt2 in VSMC Differentiation

assessed the ability of IRS-1 to recruit the p85 adapter subunit of PI3K in the presence or absence of rapamycin. We immunoprecipitated the p85 subunit of PI3K from VSMCs and immunoblotted for associated IRS-1 using an anti-phospho-tyrosine antibody (PY20). Only a single specific band migrating at the molecular weight of IRS-1 was detected with the PY20 antibody (Fig. 4D). This experiment revealed that IRS-1 was not associated with p85 PI3K in vehicle-treated (synthetic phenotype) VSMCs but that treatment with rapamycin for 1 or 24 h, or positive control treatment with insulin, promoted association of p85 with tyrosine-phosphorylated IRS-1, indicating that rapamycin promotes a functional change in IRS-1 phosphorylation such that it can recruit and activate PI3K (Fig. 4D). Controls are presented in Fig. 4E indicating that total levels of p85 PI3K do not change but that IRS-1 total protein levels and Akt activation are regulated as described in Fig. 4 (A–C).

Notably, in Fig. 4 (JOURNAL OF BIOLOGICAL CHEMISTRY 36116), we investigated whether this may be the mechanism by which mTOR inhibition activates Akt. We infected VSMCs with a control adenovirus or with adenovirus expressing rapamycin-resistant S6K1 (ED3E mutant) (7) and assessed Akt activation and differentiation marker expression. The S6K1-ED3E mutant exhibited rapamycin-resistant activity as expected, as measured by partial rescue of ribosomal S6 protein phosphorylation in the presence of rapamycin (Fig. 5). This experiment revealed that S6K1-ED3E prevented the rapamycin-induced Akt activation, mobility shift, and stabilization of IRS-1 (Fig. 5A), as well as induction of contractile proteins (Fig. 5B), suggesting that relieving S6K1 inhibition of the IRS-1/PI3K/Akt pathway is an essential signaling event for rapamycin-induced differentiation of VSMCs.

Rapamycin-induced VSMC Differentiation Requires Akt2—We next investigated whether Akt is the PI3K effector necessary for rapamycin-induced differentiation using an siRNA strategy. To determine which Akt isoform is required, we employed siRNA to specifically knockdown Akt1 or Akt2 expression. Akt3 has a limited tissue distribution (28), and we were unable to detect Akt3 expression at the protein level by Western blotting with commercially available Akt3-specific antibodies and high sensitivity enhanced chemiluminescence reagents (data not shown). siRNA inhibited expression of Akt1 protein by 76% (Fig. 6B) or 59% (Fig. 6B) and Akt2 protein by 57% (Fig. 6A) or 78% (Fig. 6B). Akt2 knockdown slightly decreased Akt1 expression, but Akt1 knockdown enhanced expression of Akt2. Surprisingly, Akt1 knockdown induced basal calponin expression, which was not further augmented by rapamycin (Fig. 6, A and B), suggesting that Akt1 exerts a repressive effect on contractile expression. Knockdown of Akt2, conversely, inhibited rapamycin-induced expression of multiple differentiation markers at the protein or mRNA level, including calponin, h-caldesmon, of IRS-1 serine 636 phosphorylation correlates with the induction of tyrosine phosphorylation.

We have previously shown that overexpression of rapamycin-resistant S6K1 prevents rapamycin-induced VSMC differentiation (7). Because S6K1 has been shown to be the mTOR effector that mediates inhibitory phosphorylation of IRS-1 in other cell types, and because S6K1 inhibition correlates temporally with Akt activation (Figs. 1–4), we investigated whether this may be the mechanism by which mTOR inhibition activates Akt. We infected VSMCs with a control adenovirus or with adenovirus expressing rapamycin-resistant S6K1 (ED3E mutant) (7) and assessed Akt activation and differentiation marker expression. The S6K1-ED3E mutant exhibited rapamycin-resistant activity as expected, as measured by partial rescue of ribosomal S6 protein phosphorylation in the presence of rapamycin (Fig. 5). This experiment revealed that S6K1-ED3E prevented the rapamycin-induced Akt activation, mobility shift, and stabilization of IRS-1 (Fig. 5A), as well as induction of contractile proteins (Fig. 5B), suggesting that relieving S6K1 inhibition of the IRS-1/PI3K/Akt pathway is an essential signaling event for rapamycin-induced differentiation of VSMCs.
and MHC (Fig. 6, A, B, D, and E), revealing that Akt2 is necessary for contractile protein induction.

The samples in which Akt1 or Akt2 expression was knocked down were examined for Akt phosphorylation at serine 473. Because Akt phosphorylation at serine 473 persisted in cells in which Akt1 was knocked down, but was lost in cells in which Akt2 was knocked down, this assay revealed that rapamycin did not appreciably activate Akt1, but predominantly activated the Akt2 isoform (Fig. 6, A–C). Fig. 6C shows averaged data from four separate experiments confirming that Akt2 is necessary for both basal and rapamycin-induced differentiation, whereas Akt1 may play an inhibitory role in contractile protein regulation and is not activated by rapamycin treatment.

To confirm our results obtained with depletion of Akt isoforms, we overexpressed wild-type Akt1 or Akt2, or constitutively active myristoylated Akt2 (myr-Akt2) in human VSMCs. Consistent with our findings in Fig. 6, overexpression of wild-type Akt2 or myr-Akt2 was sufficient to induce contractile protein expression (SM-MHC, calponin, and SM-α-actin) in the absence of rapamycin (Fig. 7). Notably, even high levels of Akt1 activity due to overexpression failed to induce contractile protein expression. Our studies reveal an isoform-specific effect of Akt in VSMC differentiation. Akt2 activity is both necessary and sufficient for contractile protein expression. In contrast, Akt1 is neither necessary nor sufficient and may even oppose VSMC differentiation.

**DISCUSSION**

This study provides several important new insights into the mechanisms of rapamycin-mediated signal transduction in VSMCs that leads to favorable phenotypic modulation. Our experiments have revealed that Akt2 activation is necessary for rapamycin-induced VSMC differentiation. We have previously shown that inhibition of the mTOR effector p70 S6K1 is necessary for rapamycin to promote the contractile phenotype (7). Here, we find that inhibition of IRS-1 and its subsequent signaling to Akt2 is one mechanism by which S6K1 opposes VSMC differentiation. This is the first demonstration of this feedback loop in which mTORC1, via IRS-1, attenuates PI3K signaling in vascular smooth muscle. Notably, our findings reveal a critical role specifically for the Akt2 isoform in VSMC phenotypic modulation.

**mTOR/S6K1 Feedback Regulation of the PI3K Pathway**—mTORC1 integrates multiple signals to provide a critical checkpoint function for cellular protein synthesis, and rapamycin mimics nutrient starvation by inhibiting this complex (9). In other insulin-responsive tissues, nutrient-dependent mTOR activity prevents excessive insulin-dependent PI3K signaling (10). This homeostatic feedback is mediated by S6K1 phosphorylation of serine residues in IRS-1. Although IRS-1 contains more than 50 consensus motifs for Ser/Thr phosphorylation (27), Ser-302, Ser-307, and Ser-636/639 have been implicated as S6K1 targets (24–26). Phosphorylation of Ser-302 or Ser-307 can inhibit IRS-1 activity in two ways. This phosphorylation inhibits IRS-1 association with the receptor tyrosine kinase, thus inhibiting the receptor-mediated tyrosine phosphorylation of IRS-1 that recruits the p85 subunit of PI3K. Importantly, this S6K1-dependent serine phosphorylation also targets IRS-1 for degradation (10). Ser-636/639 phosphorylation of IRS-1 correlates with insulin resistance and S6K1 activation (10). Additionally, mTOR itself may phosphorylate Ser-636/639, and this includes recruitment of p85 PI3K to IRS-1-phospho-Tyr-612 (29). Our data provide multiple lines of evidence that mTOR- and S6K1-mediated feedback regulation of IRS-1 occurs in vascular smooth muscle and plays an important role in modulating the VSMC phenotype (see model, Fig. 8).

mTOR feedback regulation of PI3K signaling via IRS-1 has been identified as a potential mechanism underlying insulin resistance in type II diabetes (12). S6K1 deletion protects mice...
from diet-induced obesity and confers hypersensitivity to insulin (26), and amino acid stimulation of mTOR inhibits insulin response in skeletal muscle in humans (11). Diabetic patients suffer accelerated vascular disease, including atherosclerosis, and more severe restenosis (31). Our work therefore suggests that the mTOR inhibitory regulation of IRS-1 may contribute to diabetic complications of the vasculature in part by modulating VSMC phenotype. Notably, rapamycin-eluting stents have improved revascularization outcomes for diabetic patients. In recent trials, rapamycin-eluting stents provided an advantage over paclitaxel-eluting stents in inhibiting restenosis (2, 3) that was particularly apparent in diabetic patients (31). Although both drugs inhibit VSMC proliferation, it is tempting to speculate that rapamycin may additionally improve insulin- and IGF-sensitivity and promote VSMC differentiation through IRS-1 stabilization.

It was recently discovered that mTOR, in complex with rictor and mLST8 (mTORC2), is the PDK2 activity that phosphorylates Akt at Ser-473 (32). This complex is acutely rapamycin insensitive but can be inhibited by chronic treatment with rapamycin in some cell types (32). Our data reveal a partial diminution of Akt phosphorylation after 24 h rapamycin in some experiments (Fig. 1). This is consistent with the partial inhibition of Akt after 24-h rapamycin reported in rat VSMCs (33). The efficacy of rapamycin in vivo suggests that transient activation of Akt after injury may be sufficient to inhibit intimal hyperplasia. In contrast, Akt was strongly inhibited in endothelial cells (human umbilical vein endothelial cells) after 24-h rapamycin (33), suggesting the possibility that mTOR/rictor inhibition may contribute to the recently described increased risk of thrombosis conferred by rapamycin-eluting stents (4). Elucidation of the VSMC-specific pro-differentiation targets of rapamycin may lead to development of improved targeted therapeutics that spare endothelial cell survival.

Interestingly, a recent report suggested that the mTOR pathway can also negatively regulate platelet-derived growth factor signaling, at the level of receptor expression. Rapamycin activated Akt and increased expression of the platelet-derived growth factor receptor in TSC-deficient cells (30). However, the mechanism does not appear to be inhibition of platelet-derived growth factor receptor degradation as is the case for IRS-1, but an effect at the mRNA level. It will be of interest to determine the extent to which mTOR/S6K1 negative feedback may regulate other growth factor-signaling pathways.

mTOR, S6K1, and VSMC Differentiation—One hallmark of the dedifferentiated “synthetic” VSMC phenotype is high levels of extracellular matrix synthesis that contributes to intimal hyperplastic lesions (1). mTORC1 modulates protein synthesis through its effectors S6K1 and 4EBP1 (8), and we reported that
mTOR inhibition with rapamycin inhibited total protein and collagen synthesis in VSMCs (7). We further found that rapamycin-resistant S6K1 prevented rapamycin-induced expression of contractile proteins (7). Here, we show that the role of S6K1 in VSMC phenotypic modulation is not limited to protein synthesis but that it negatively regulates the pro-differentiation p70 S6K1/2 activity in VSMCs. It is likely that S6K1 may have other substrates besides IRS-1 that influence VSMC differentiation. S6K1 is necessary for rapamycin-sensitive migration in other cell types, suggesting an additional mechanism by which S6K1 in VSMC phenotypic modulation is not limited to protein synthesis but that it negatively regulates the pro-differentiation p70 S6K1/2 activity in VSMCs. It is likely that S6K1 may have other substrates besides IRS-1 that influence VSMC differentiation. S6K1 is necessary for rapamycin-sensitive migration in other cell types, suggesting an additional mechanism by which S6K1 may have other substrates besides IRS-1 that influence VSMC differentiation.

IGF-I Signaling in VSMC Differentiation—Our data, showing that a neutralizing antibody to IGF-I completely blocks rapamycin-induced Akt activation, and that IGF-I addition to serum-free media reconstitutes both rapamycin-induced Akt activation and contractile protein expression, suggest that IGF-I is the essential serum growth factor that drives VSMC differentiation in our model. There is strong evidence for IGF-I as a pro-differentiation factor in SMCs (13–15). Interestingly, IGF-I has been implicated in VSMC proliferation and migration (35), as well as in differentiation. This dichotomy has been addressed by Sobue et al., who demonstrated that IGF-I maintains VSMC differentiation by preferential activation of p70 S6K1 versus ERK1/2 (13, 14), with selective p70 S6K1 activation mediated by IRS-1 recruitment of SHP-2 (36). In dedifferentiated VSMCs, SHP-2 did not associate with IRS-1, and IGF-I instead potently activated ERK1/2, resulting in proliferation and migration. Although we did not examine SHP-2 association with IRS-1, our data that rapamycin and IGF-I promote differentiation via Akt suggest that SHP-2 is likely recruited to IRS-1 under these conditions. It will be of interest to determine whether rapamycin regulates SHP-2 association with IRS-1 in future experiments.

The high levels of growth factors at a site of vascular injury are known to induce VSMC proliferation and migration, via ERK and other signals (1). We hypothesize that, in addition to promoting protein synthesis, migration, and proliferation, the enhanced growth factor stimulation of mTORC1/S6K1 inhibits the expression of contractile proteins. The augmentation of IGF-I/p70 S6K1/Akt signaling by rapamycin may induce VSMC differentiation in a lesion.

Akt Isoforms—Our data reveal that Akt isoforms serve distinct functions in VSMCs, and that only Akt2 is activated by rapamycin and required for rapamycin-induced differentiation. Interestingly, selective inhibition of Akt1 with pharmacologic inhibitors or dominant negative Akt1 failed to inhibit rapamycin-induced VSMC differentiation. Furthermore, overexpression of Akt2, but not Akt1, was sufficient to induce contractile protein expression. Our data, implicating Akt2 as the critical IGF-I effector in differentiation signaling, are consistent with the elegant work of Birnbaum et al. (18, 19) who have demonstrated that Akt1 and Akt2 perform non-redundant physiological functions in knock-out mice: Akt1 regulates animal size, but Akt2 is required for insulin-dependent glucose homeostasis. Others have found distinct roles for Akt1 and Akt2 in IGF-I signaling in phenotypic modulation in mammary epithelial cells (20). A recent study revealed opposing functions for these Akt isoforms, with Akt1 promoting, but Akt2 inhibiting migration in mouse embryo fibroblasts (21). This suggests the intriguing possibility that Akt2 activation may be the mechanism by which rapamycin inhibits the VSMC migration that occurs in restenosis.

Of the three Akt isoforms, Akt1 and Akt2 are ubiquitously expressed, whereas Akt3 is found primarily in the brain (28). Because these isoforms share a high degree of sequence homology, it is unclear what accounts for the differences in substrate specificity. Interestingly, in adipocytes where Akt2, but not Akt1, activity correlates with differentiation, these isoforms are localized to distinct compartments (37, 38). Our preliminary data suggest that Akt1 and Akt2 also have different relative subcellular distributions in VSMCs. We are actively investigating the localization, regulation, and functions of these Akt isoforms in VSMCs.

Work by others has suggested potential Akt substrates that may regulate VSMC differentiation, including FOXO family transcription factors (39) and calcineurin (15). We are currently investigating these and other potential Akt targets as effectors of rapamycin-induced differentiation.

Because Akt and S6K1 are both p70 S6K1-dependent kinases (8), both are normally activated by growth factor stimulation. Notably, mTORC1 inhibition by nutrient starvation or with rapamycin provides a unique signaling pattern where one p70 S6K1 effector, Akt, is activated, but another, S6K1, is inhibited. We hypothesize that this combination results in the optimal signal for promotion of the differentiated smooth muscle phenotype.
Rapamycin Activates Akt2 in VSMC Differentiation

and may explain the utility of rapamycin as a vascular therapeutic. Rapamycin-activated Akt has the added advantage of promoting VSMC survival, not apoptosis, and induces a quiescent, contractile, non-migratory phenotype characteristic of mature, differentiated smooth muscle cells.

In conclusion, we show that rapamycin inhibits S6K1-mediated repression of IRS-1, leading to Akt2 activation and VSMC differentiation. This study provides further insights into the molecular signaling mechanisms by which rapamycin promotes VSMC differentiation, revealing for the first time the existence of mTOR/P13K feedback that may have important implications for understanding the pathogenesis of vascular disease and diabetes.

Acknowledgments—We thank Dr. John Blenis for critically reviewing the manuscript and for the gift of anti-HA antibody. We thank Dr. Jin Q. Cheng for the gift of Akt expression plasmids. We thank Trisha Noreault and Lauren Wasson for technical assistance.

REFERENCES

1. Owens, G. K., Kumar, M. S., and Wamhoff, B. R. (2004) Physiol. Rev. 84, 767–801
2. Moliterno, D. J. (2005) N. Engl. J. Med. 353, 724–727
3. Eisenberg, M. J., and Konnyu, K. J. (2006) Am. J. Cardiol. 98, 375–382
4. Stone, G. W., Moses, J. W., Ellis, S. G., Schofer, J., Dawkins, K. D., Morice, M. C., Colombo, A., Schampaert, E., Grube, E., Kirtane, A. J., Cutlip, D. E., Fahy, M., Pocock, S. J., Mehran, R., and Leon, M. B. (2007) N. Engl. J. Med. 356, 998–1008
5. Poon, M., Marx, S. O., Gallo, R., Badimon, J. J., Taubman, M. B., and Marks, A. R. (1996) Circulation 94, 2277–2283
6. Marx, S. O., Jayaraman, T., Go, L. O., and Marks, A. R. (1995) Circ. Res. 76, 412–417
7. Martin, K. A., Rzucidlo, E. M., Merenick, B. L., Finger, D. C., Brown, D. J., Wagner, R. J., and Powell, R. J. (2004) Am. J. Physiol. 286, C507–C517
8. Martin, K. A., and Blenis, J. (2002) Adv. Cancer Res. 86, 1–39
9. Sarbassov, D. D., Ali, S. M., and Sabatini, D. M. (2005) Curr. Opin. Cell Biol. 17, 596–603
10. Harrington, L. S., Findlay, G. M., and Lamb, R. F. (2005) Trends Biochem. Sci. 30, 35–42
11. Tremblay, F., Krebs, M., Dombrowski, L., Brehm, A., Bernroider, E., Roth, E., Nowotny, P., Waldbaum, W., Marette, A., and Roden, M. (2005) Diabetes 54, 2674–2684
12. Um, S. H., D’Alessio, D., and Thomas, G. (2006) Cell Metab. 3, 393–402
13. Hayashi, K., Saga, H., Chimori, Y., Kimura, K., Yamanaka, Y., and Sobue, K. (1998) J. Biol. Chem. 273, 28860–28867
14. Hayashi, K., Takahashi, M., Kimura, K., Nishida, W., Saga, H., and Sobue, K. (1999) J. Cell Biol. 145, 727–740
15. Ohkawa, Y., Hayashi, K., and Sobue, K. (2003) Biochem. Biophys. Res. Commun. 301, 78–83
16. Brown, D. J., Rzucidlo, E. M., Merenick, B. L., Wagner, R. J., Martin, K. A., and Powell, R. J. (2005) J. Vasc. Surg. 41, 509–516
17. Hixon, M. L., Muro-Cacho, C., Wagner, M. W., Obeijero-Paz, C., Millie, E., Fujiy, Y., Kureishi, Y., Hassold, T., Walsh, K., and Gualberto, A. (2000) J. Clin. Invest. 106, 1011–1020
18. Cho, H., Thrvaldsen, J. L., Chu, Q., Feng, F., and Birnbaum, M. J. (2001) J. Biol. Chem. 276, 38349–38352
19. Cho, H., Mu, J., Kim, K., Thrvaldsen, J. L., Chu, Q., Crenshaw, E. B., 3rd, Kaestner, K. H., Bartolomei, M. S., Shulman, G. M., and Birnbaum, M. J. (2001) Science 292, 1728–1731
20. Irie, H. Y., Pearline, R. V., Gruenenberg, D., Hsia, M., Ravichandran, P., Kothari, N., Natesan, S., and Brugge, J. S. (2005) J. Cell Biol. 171, 1023–1034
21. Zhou, G. L., Tucker, D. F., Bae, S. S., Bhatheja, K., Birnbaum, M. J., and Field, J. (2006) J. Biol. Chem. 281, 36443–36453
22. Fetalvero, K. M., Shyu, M., Nomikos, A. P., Chiu, Y. F., Wagner, R. J., Powell, R. J., Hwa, J., and Martin, K. A. (2006) Am. J. Physiol. 290, H1337–1344
23. Tremblay, F., Martere, A. (2001) J. Biol. Chem. 276, 38052–38060
24. Harrington, L. S., Findlay, G. M., Gray, A., Tolkacheva, T., Wkigfield, S., Rebholz, H., Barnett, J., Leslie, N. R., Cheng, S., Shepherd, P. R., Gout, L., Downes, C. P., and Lamb, R. J. (2004) J. Cell Biol. 166, 213–223
25. Ozes, O. N., Akca, H., Mayo, I. D., Gustin, I. A., Maehama, T., Dixon, J. E., and Donner, D. B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4640–4645
26. Um, S. H., Frigerio, F., Watanabe, M., Picard, F., Joaquim, M., Sticker, M., Furnagulli, S., Allegrini, P. R., Kozma, S. C., Auwerx, J., and Thomas, G. (2004) Nature 431, 200–205
27. Greene, M. W., and Garofalo, R. S. (2002) Biochemistry 41, 7082–7091
28. Yang, Z. Z., Tschopp, O., Hemmings-Mieszczak, M., Feng, J., Brodbeck, D., Perentes, E., and Hemmings, B. A. (2003) J. Biol. Chem. 278, 32124–32131
29. Tzatsos, A., and Kandror, K. V. (2006) Mol. Cell Biol. 26, 63–76
30. Zhang, H., Bajraszewski, N., Wu, E., Wang, H., Museman, A. P., Dabara, S. L., Griffin, J. D., and Kwiatkowski, D. J. (2007) J. Clin. Invest. 117, 730–738
31. Dilba, A., Kastrati, A., Mehilli, J., Pache, J., Schuhen, H., von Beckerath, N., Ulm, K., Wessler, R., Dirschinger, J., and Schomig, A. (2005) N. Engl. J. Med. 353, 663–670
32. Garofalo, R. S. (2002) Science 297, 663–670
33. Sabassov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005) Science 307, 1098–1101
34. Sabassov, D. D., Ali, S. M., Sengupta, S., Sheen, J. H., Hsu, P. P., Bagley, A. F., Markhard, A. L., and Sabatini, D. M. (2006) Mol. Cell 22, 159–168
35. Liu, L., Li, F., Cardelli, J. A., Martin, K. A., Blenis, J., and Huang, S. (2006) Oncogene 25, 7029–7040
36. Duan, C., Bauchat, J. R., and Hsieh, T. (2000) Circ. Res. 86, 15–23
37. Hayashi, K., Shibata, K., Morita, T., Iwasaki, K., Watanabe, M., and Sobue, K. (2004) J. Biol. Chem. 279, 40807–40818
38. Calera, M. R., Martinez, C., Liu, H., Jack, A. K., Birnbaum, M. J., and Pilch, P. F. (1998) J. Biol. Chem. 273, 7201–7204
39. Bae, S. S., Cho, H., Mu, J., and Birnbaum, M. J. (2003) J. Biol. Chem. 278, 49530–49536
40. Liu, Z. P., Wang, Z., Yanagisawa, H., and Olson, E. N. (2005) Dev. Cell 9, 261–270