Care for patients with symptomatic or unstable coronary artery disease has undergone a major evolution over the past 5 years, with shifts toward increased use of percutaneous stent implantation at the expense of thrombolytic therapy and coronary artery bypass grafting. Since the introduction of coronary artery angioplasty in the late 1970s by Andreas Greuntzig, the development of percutaneous coronary interventions has been punctuated by several major breakthroughs, including those that have transformed the field (eg, the use of stents to prevent vessel recoil) and some that have not (percutaneous atherectomy being a notable example). Although each of the major advances in interventional cardiology has been considered a threat to surgical approaches, restenosis has not been eliminated, and many questions remain about the optimal technology platform for percutaneous interventions.

Restenosis after percutaneous interventions has 2 principle components: vessel remodeling and intimal hyperplasia. Stent deployment essentially obviates the problem of remodeling by preventing chronic recoil mechanisms.2 Drug-eluting stents have been developed on the premise that pharmacological approaches using local delivery can efficiently reduce neointimal hyperplasia. At the present time, the principle of polymer-based elution of rapamycin and paclitaxel has been used on stents that are now commercially available and that have been definitively shown to reduce restenosis rates and target lesion revascularization compared with bare metal stents in large multicenter trials.3,4 Both of these drugs have been shown to inhibit smooth muscle cell migration and proliferation in vitro,5,6 but their choice over other agents with similar properties has been made partly for practical reasons. A rational method for determining the optimal pharmacological agent for drug-eluting stents has not been clearly defined. In addition, as more data accumulate, it is becoming more apparent that differences exist between available drug-eluting stent formulations. In particular, the issue has been raised that...

Objective—Advances in stent technology have enabled the delivery of drugs to improve outcomes after stent deployment. However, the optimal payloads for stents are not clear, and the appropriate stent-based therapies for high-risk patients, such as diabetics, have not been clearly established.

Methods and Results—We used smooth muscle cell culture models to compare the activities of rapamycin and paclitaxel. Smooth muscle cells were grown in normal or high glucose to induce insulin resistance. Both paclitaxel and rapamycin activate mitogen-activated protein kinase pathways similarly. However, rapamycin potently activates AKT-dependent signaling, an effect that overrides the downregulation of this pathway by insulin resistance and that causes phosphorylation of the AKT-dependent transcription factor FOXO1. This effect is associated with attenuation of the anti-migratory effects of rapamycin under high glucose conditions that are not observed with paclitaxel, as well as with increased protection against ceramide-induced cytotoxicity, both of which are dependent on FOXO1 phosphorylation.

Conclusions—Differences between the ability of rapamycin and paclitaxel to activate AKT may account for their differential cell survival and antichemotactic activities. These observations may provide a basis for understanding clinical differences between rapamycin- and paclitaxel-coated stents. The approaches used in these studies can be expanded to other candidate stent payloads as a method for triage in preclinical studies. (Arterioscler Thromb Vasc Biol. 2006;26:1473-1480.)

Key Words: migration ■ signaling ■ smooth muscle ■ stent ■ viability
certain high risk groups such as diabetics may have different target lesion revascularization rates depending on the drug that is delivered, and the most appropriate stent formulation for these and other high risk patients remains controversial.1,3,4,7,8

Paclitaxel, semisynthetic diterpenoid, binds the ®-tubulin subunit of microtubules, facilitating their polymerization and preventing their depolymerization. This effect prevents the re-organization of microtubules into the mitotic spindle, arresting the cell cycle at the G1 and G2/M junctions.9 Moreover, as dynamic microtubule remodeling is necessary for proper cytoskeletal architecture, paclitaxel is a potent inhibitor of cell migration as well as proliferation. Paclitaxel also has a wide range of additional effects that are not well understood and are not obviously related to its effects on mitosis and cell mobility. In contrast, rapamycin (sirolimus) is a macrocyclic lactone that engages FK506-binding protein 12, inhibiting the activation of mTOR, a crucial cell-cycle regulatory protein. Inhibition of the mTOR pathway blocks cellular transition from the G1 to S phase, effectively stopping mitosis.10 Rapamycin also has potent anti-cellular migration activity through a mechanism that is not fully understood.11 However, the comparative effects of these drugs in smooth muscle cells have not been carefully studied. Therefore, we have used a cell culture system that allows us to perform comparative analyses of drugs on smooth muscle cell phenotypes that are implicated in the pathophysiology of in-stent restenosis. This system also allows us to mimic conditions such as insulin resistance that may modify the response to stent deployment in vivo. Using this approach, we have found that differences exist in the activities of rapamycin and paclitaxel toward smooth muscle cells that may contribute to differences in outcomes after drug-eluting stent implantation.

Materials and Methods

Cell Culture
Rat aortic smooth muscle cells (RASMC) were isolated from the thoracic aortas of 200-gram to 250-gram male Harlan Sprague-Dawley rats by the explant method as previously described.11 Cultures prepared under these conditions were routinely smooth muscle actin-positive (>95%) and platelet-endothelial cell adhesion molecule-negative by immunofluorescence after 2 passages, and the studies here were performed within 6 passages from explant. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% (volume/volume) fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 µg/mL Amphotericin-B at 37°C in 5% CO2. All experiments were conducted using RASMC that were quiesced by incubation in DMEM containing 0.1% calf serum for 72 hours. Cells were maintained from explant in 5 mmol/L glucose and were placed in 25 mmol/L glucose (or 25 mmol/L mannitol as a control for osmolality) for 72 hours before experiments were initiated when indicated. RASMC were transfected with pFOXO1A3 or the empty vector pECHA using LOF liposomes as previously described.13

Western Blot Analysis
Quiescent RASMC were treated in the presence and absence of agonists and/or inhibitors for the indicated times. Cells were then scraped into 1.5 mL tubes. The lysates were placed on ice for 15 minutes and then centrifuged at 12 000 rpm for 20 minutes. Cellular protein (50 µg) was resolved by electrophoresis on a 0.1% SDS–10% polyacrylamide gel (SDS-PAGE) under denaturing conditions. The proteins were transferred electrothermally to polyvinylidene fluoride membranes (Immobilon-P; Millipore). After blocking in 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% Tween-20 (TBS-T), and 5% (wt/vol) nonfat dry milk, the membranes were treated with primary antibodies overnight at 4°C with constant agitation. All antibodies (both phospho-specific and total protein) were purchased from Cell Signaling Technologies, except for phospho-IRS-1 pY612, which was purchased from AbCam. Membranes were then washed 3 times at room temperature in TBS-T, followed by incubation with peroxidase-conjugated secondary antibodies diluted 1:1000 in TBS-T containing 5% nonfat dry milk. The immunocomplexes were detected using a chemiluminescence reagent kit (Amersham Corp) and recorded by exposure to Hyperfilm ECL (Amersham). Solutions of rapamycin and paclitaxel (LC Laboratories) and LY294002 and ceramide (Cayman Chemical) were prepared in DMSO at the indicated concentrations. Blots were quantified by densitometry and quantitative analyses include at least 3 replications per condition.

Haptotactic Cell Migration Assay
Haptotactic cell migration assays were performed using modified Boyden chambers (6.5-mm polycarbonate transwell filter inserts with 8-µm pores, Transwell; Costar). The bottom side of the filters was coated with 5 µg/mL vitronectin. Transwell filters were air-dried for 1 hour in a tissue culture hood. The filters were washed with PBS and blocked with 2% bovine serum albumin in PBS. Growth-arrested RASMC were trypsinized, centrifuged, and resuspended in DMEM without serum. RASMC were treated or without 100 mmol/L insulin for 30 minutes, and 2×106 cells in 100 µL of DMEM were seeded onto the top of the Transwell filters. Cells were incubated at 37°C for 12 hours. The wells were washed twice with PBS, the cells from the top side of the filter were removed with a cotton swab, and cells on the bottom side of the filter were stained with hematoxylin. The number of blue cells in a 10×10-mm grid were scored for 6 fields (20× objective) per filter. The average number of cells that migrated per filter was calculated.

Cell Survival Assay
Cell survival assays were performed in triplicate in 6-well culture plates. RASMC (1×104) were plated in each well and grown to 80% confluence in DMEM supplemented with 10% FBS, 5.5 mmol/L glucose, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO2-enriched humidified atmosphere. Cells were quiesced in medium containing 0.1% FBS for 72 hours. Twenty-four hours before drug treatment, cells were re-fed with medium plus 0.1% FBS containing either 5 mmol/L or 25 mmol/L glucose. Cells were treated with 5 µg/mL C2-ceramide (Calbiochem) and varying doses of rapamycin, paclitaxel, or vehicle. RASMC were incubated at 37°C for 60 minutes, and then treated with 100 mmol/L insulin for 48 hours. Total and viable cell counts were determined by Tripan blue exclusion, and cell survival was expressed as a percentage representing the ratio of viable to total cells.

Statistical Analysis
Data are representative of at least 3 independent experiments and quantitative analyses are presented as mean±SEM. Significance was evaluated using Student t test. A value of P<0.05 was considered statistically significant.

Results
Modeling Insulin Resistance in Rat Aortic Smooth Muscle Cells
As a first step to establish cell culture conditions that mimic insulin resistance, we adapted previously described approaches by growing cultured RASMC under high
Effects on Mitogen-Activated Kinase Signaling

As a first step to characterize potential differences between rapamycin and paclitaxel at the level of cell signaling in smooth muscle cells, we performed a survey of signaling pathway activation. Activation of mitogen-activated kinases signaling pathways has been linked to migratory and proliferative events downstream of growth factors and insulin.\(^\text{14}\) We tested rapamycin and paclitaxel in concentrations of 0.01 to 1 ng/mL, which overlaps the range of concentrations expected in vivo.\(^\text{15}\) We found that both rapamycin and paclitaxel (0.1 to 1 ng/mL) hyperactivated ERK1/2 activity at low doses after insulin stimulation (data not shown), which is consistent with previous observations of mitogen-activated protein (MAP) kinase activation by these agents in cancer cell lines.\(^\text{16}\) Both paclitaxel and rapamycin had little consistent effect on p38 MAP kinase activation (data not shown), indicating that with respect to activation of MAP kinase pathways, these drugs behaved similarly in vascular smooth muscle cells.

Rapamycin Induces AKT Activation in Smooth Muscle Cells

Because we did not identify any differences in the effects of rapamycin and paclitaxel on MAP kinase activation in smooth muscle cells, we tested activation of the phosphatidylinositol 3-kinase/Akt kinase/mTOR pathway based on links between these signal events and smooth muscle cell migration and survival.\(^\text{17,18}\) Neither rapamycin nor paclitaxel had any appreciable effect on insulin-dependent IRS-1 phosphorylation under any conditions (data not shown). However, when we examined AKT, we found that whereas paclitaxel pretreatment for 1 hour had no effect on phosphorylation of AKT at serine 473 after insulin stimulation at concentrations of 0.01 to 1 ng/mL (and potently suppressed AKT phosphorylation at doses of 100 ng/mL or greater, see supplemental Figure IA, available online at http://atvb.ahajournals.org) rapamycin potently and surprisingly resulted in AKT phosphorylation at a concentration threshold of 0.1 ng/mL (Figure 2A and 2B). This effect was relatively more potent under high glucose conditions; however, whereas insulin resistance otherwise suppressed insulin-induced AKT activation, rapamycin still elicited 2.5-fold increase in phospho-AKT levels at concentrations of 0.01 ng/mL. Identical results were obtained when phosphorylation of an alternative residue involved in AKT activation, threonine 308, was tested (data not shown).

To determine whether these effects of rapamycin were mediated via phosphatidylinositol 3-kinase, we pretreated smooth muscle cells with Ly294002, a potent inhibitor of phosphatidylinositol 3-kinase, for 1 hour before adding rapamycin and insulin. Ly294002 inhibited insulin-induced AKT phosphorylation, which is known to be phosphatidylinositol 3-kinase–dependent, whereas Ly294002 pretreatment had no effect on rapamycin-induced AKT activation, indicating that rapamycin’s ability to activate AKT is independent of phosphatidylinositol 3-kinase (Figure 2C).

To assess the consequences of AKT phosphorylation induced by rapamycin, we measured the activation of known downstream targets of the AKT pathway. mTOR activity, as measured by phosphorylation of p70 S6 kinase on residue threonine 389, was inhibited by rapamycin at concentrations of 0.1 ng/mL or greater, as would be expected (Figure 3A); paclitaxel had no effect on mTOR activity at any dose (Figure 3B). We also examined the activity of FOXO1, a transcription factor that is phosphorylated and inactivated by AKT.\(^\text{19}\) Consistent with its ability to induce AKT phosphorylation, the abundance of phospho-FOXO1 was also enhanced by rapamycin by AKT, whereas paclitaxel had no effect on FOXO1 activation at any dose (Figure 3C), indicating that activation of AKT by rapamycin leads to further modulation of downstream signaling pathways in RASMC.

Figure 1. Insulin resistance induced by high glucose in rat aortic smooth muscle cells. A to C, Western blot analysis was used to test insulin-induced (100 nmol/L) phosphorylation of IRS-1 and AKT in smooth muscle cells cultured in low-glucose (NG; 5 mmol/L) or high-glucose (HG; 25 mmol/L) conditions. A, Insulin-induced phosphorylation of IRS-1 was both delayed and diminished in high-glucose cultured cells over time. B, Quantitative analysis of IRS-1 phosphorylation after 5 minutes of stimulation was determined by densitometry and presented as the mean±SD (n=3). The difference in IRS-1 phosphorylation between NG and HG conditions was significant at P<0.01. C, Insulin-induced phosphorylation of AKT was also diminished in high-glucose cultured cells.

(25 mmol/L) or normal (5 mmol/L) glucose conditions for 72 hours. Importantly, we noted that most standard smooth muscle cell media preparations include relatively high concentrations of glucose, and to minimize long-lasting changes in insulin signaling, smooth muscle cells were cultured in 5 mmol/L glucose from the explant stage, and then were either maintained in low glucose or switched to high glucose conditions before performing experiments. Under these conditions, we found that insulin-dependent phosphorylation of IRS-1 and AKT, which are key downstream targets in the insulin signaling pathway, was markedly attenuated under high glucose conditions in smooth muscle cells (Figure 1A through 1C), indicative of insulin resistance under these conditions.
Differential Effects of Rapamycin by Platelet-Derived Growth Factor-Dependent Signaling

Our initial evaluation focused on the effects of rapamycin and paclitaxel under conditions of insulin stimulation, based on the known effects of insulin on enhancing smooth muscle cell migration and survival. To determine whether the effects observed on signaling pathways were generalizable or specific to insulin-dependent signaling, we tested whether similar patterns were observed after platelet-derived growth factor (PDGF)-BB stimulation. We found that PDGF was a more potent activator of extracellular signal regulated kinase (ERK) and AKT than insulin in RASMC, and that rapamycin and paclitaxel had little effect on these pathways at the concentrations used (data not shown). In particular, rapamycin did not potentiate PDGF-dependent AKT activity under either normal or high glucose conditions, indicating that this effect occurs specifically in the context of insulin activation.

Differential Effects of Paclitaxel and Rapamycin on Smooth Muscle Cell Phenotypes

Because AKT activation is closely associated with smooth muscle cell survival, we tested whether rapamycin or paclitaxel treatment had any effect on smooth muscle cell responses to ceramide, which is known to impair smooth muscle cell survival by decreasing AKT activity. Paclitaxel had no effect on the ability of ceramide to elicit a cytotoxic response in RASMC (Figure 4A and 4B). In contrast, rapamycin reversed the effects of ceramide on cell survival over a broad dose range, consistent with its ability to induce phosphorylation of the survival factor AKT. This effect of rapamycin was observed under both normal and high glucose conditions, but the ability of rapamycin to augment cell survival in cells treated with ceramide was significantly greater at low concentrations (0.01 mg/mL) in RASMC grown in high glucose, suggesting an interaction between hyperglycemia-induced and rapamycin-dependent signaling to enhance survival.

We also examined the comparative effects of paclitaxel and rapamycin over a broad concentration on smooth muscle...
cell migration based on the close association of dysregulated migration with restenosis in vivo and with AKT activation in vitro. Under low glucose conditions, both paclitaxel and rapamycin were potent inhibitors of insulin-induced smooth muscle cell migration (Figure 4C and 4D), even at the lowest concentrations tested (0.01 ng/mL). However, in contrast with paclitaxel, the antimigratory effects of rapamycin were markedly attenuated under high glucose conditions, with

Figure 4. Comparative effects of rapamycin and paclitaxel on smooth muscle cell phenotypes. A and B, The effects of escalating doses of rapamycin (A) and paclitaxel (B) or vehicle on smooth muscle survival (percentage viability determined by Trypan blue exclusion) when administered 1 hour before ceramide treatment (5 μg/mL) were assessed in smooth muscle cells treated with insulin as indicated in 3 replicate experiments. C and D, Insulin-dependent smooth muscle cell migration was measured using modified Boyden chamber assays in insulin-stimulated cells grown under low-glucose or high-glucose conditions in the presence of escalating doses of rapamycin (C) and paclitaxel (D). Migrating cells were counted and expressed as a percentage of control. E, Cell migration was measured in smooth muscle cells grown in normal or high glucose after transfection with pFOXO1A3 or control plasmid for 24 hours, and then treated with the indicated concentrations of rapamycin and insulin. F, Cell viability was measured by Trypan blue exclusion in RASMC grown in normal or high glucose after transfection with pFOXO1A3 or control plasmid for 24 hours, and then treated with the indicated concentrations of rapamycin and insulin. Western blot analysis to demonstrate FOXO1A3 expression is shown in the lower panel.
Remarkably, the efficiency of rapamycin-mediated inhibition of insulin stimulation in cells grown in low-glucose medium. Having shown that rapamycin induces phosphorylation of FOXO1 (Figure 3C), which in turn represses FOXO1 transcriptional activity, we wanted to explore the possibility that reactivation of FOXO1 would enhance the antimitagatory effects of rapamycin underhyperglycemic conditions. To do this, we overexpressed a mutated form of FOXO1 (FOXO1A3, in which the 3 AKT phosphorylation sites are mutated) using a liposome-dependent transient transfection protocol that results in 80% to 85% transfection efficiency in RASMC. Forced expression of FOXO1A3 suppressed migration by \( \approx 20\% \) in RASMC grown in 5% glucose and had little additional effect above that of rapamycin under these conditions (Figure 4E). However, the ability of hyperglycemia to attenuate the antimitagatory effects of rapamycin was lost when FOXO1A3 was overexpressed. Similarly, we found that overexpression of FOXO1A3 reversed the pro-survival effects of rapamycin in RASMC treated with ceramide (Figure 4F). Taken together, these experiments argue for a direct role of AKT-induced phosphorylation of FOXO transcription factors in mediating the effects of rapamycin on RASMC migration and survival under different glycemic conditions.

**Discussion**

The addition of the drug-eluting stent to the interventionalist’s arsenal marks a watershed in the treatment of coronary artery disease. Whereas rapamycin- and paclitaxel-eluting stents have revolutionized the treatment of coronary artery disease, a complete understanding of the mechanisms of action of these drugs is necessary to fully realize the benefits of this new technology. Our studies have uncovered differences in the behavior of these 2 drugs in vascular smooth muscle cells that may have implications for the treatment of coronary artery disease in diabetic patient populations. Cells grown in a high glucose (25 mmol/L) environment rapidly became insensitive to insulin, producing an in vitro model of insulin resistance. Initial characterization of this model demonstrated a reduction in cellular proliferation in response to insulin, reduced cellular migration, and diminished phosphorylation of IRS and AKT (Figure 1). These results indicate that high glucose treatment mimics both behaviorally and biochemically, the smooth muscle cellular response characteristic of type II diabetes. Nevertheless, it is important to emphasize that vascular lesions contain cell types other than smooth muscle that may be relevant to vasculoproliferative responses, and that simply mimicking insulin resistance may not fully recapitulate all the factors in the type II diabetic that impact on vascular responses. Using this model system, we have dissected the molecular differences in cellular behavior and signal pathway alterations induced by rapamycin and paclitaxel.

Phenotypically, both drugs act over a broad range of concentrations to reduce cellular migration in response to insulin stimulation in cells grown in low-glucose medium. Remarkably, the efficiency of rapamycin-mediated inhibition of migration is markedly diminished in a high-glucose environment (Figure 4). In the in vivo situation, smooth muscle cell migration into the neointima may accelerate the restenotic process after PCI. Analysis of the signaling pathways modulated by paclitaxel and rapamycin reveal several insights into the mechanism of action of these drugs in vascular smooth muscle cells.

MAP kinase signaling pathways were activated by both drugs after insulin stimulation in smooth muscle cells, an effect that has been observed in other cell types but that does not explain the migratory or survival effects we observed. Because of this, we characterized the effects of these drugs on the PI3 kinase/AKT kinase/mTOR signaling axis, which plays a critical role in mitogen-stimulated migration and survival in vascular smooth muscle cells and is a known target of rapamycin. We analyzed several points along this pathway to determine the effects of rapamycin and paclitaxel. Neither drug alters insulin-dependent IRS-1 phosphorylation. These results indicate that the initiation of signaling is not affected by drug treatment, and any derangements of this axis are likely to be at the level of AKT or below.

Analysis of AKT phosphorylation (which denotes its active form) indicates several important observations about the differential effects of these drugs in low- and high-glucose model systems. Measurement of AKT phosphorylation demonstrates that paclitaxel does not activate AKT under any conditions (Figure 2). Additional studies indicated that paclitaxel markedly inhibits insulin-dependent AKT phosphorylation at concentrations \( \geq 100 \) ng/mL (supplemental Figure IA). This effect is observed in both high-glucose and normal glucose environments. Unexpectedly, rapamycin induces paradoxical AKT activation at a concentration of 1 ng/mL under normal glucose conditions. Moreover, this activation of AKT by rapamycin is even more striking under conditions of insulin resistance, with phosho-AKT levels being 2.5-fold higher in cells treated with concentrations of 0.01 ng/mL. In contrast, and as expected, mTOR activity (as measured by p70 S6 kinase phosphorylation at threonine 389) is dramatically inhibited by rapamycin, and paclitaxel has no effect on mTOR activity at any dose tested. The differential effects of rapamycin at different levels of the PI3 kinase/AKT kinase/mTOR axis may therefore be caused by a feedback activation of AKT when mTOR activity is inhibited, which in turn may lead to activation of other AKT target pathways that are responsible for the paradoxical response of the vascular smooth muscle cells, especially in the setting of deranged insulin responsiveness. We have examined upstream factors that are known to regulate AKT activation and have found that neither changes in the phosphorylation status of PDK1, which is known to phosphorylate AKT, nor changes in the activity of protein phosphatase 2A, which dephosphorylates and inactivates AKT, were observed that explained the effects of rapamycin on AKT phosphorylation (supplemental Figure IB through ID). Recent studies have indicated that mTOR exists in 2 complexes, an mTOR/raptor complex that serves the canonical function of phosphorylating p70 S6 kinase and other classical mTOR targets, and an mTOR/rictor complex that is rapamycin-insensitive and that activates AKT. We tested whether this pathway might serve as a feedback mechanism to sense repression of mTOR by rapha-
mycin and elicit activation of AKT, and whereas this model would be entirely consistent with the rapamycin-dependent activation of AKT we observe, we have not yet been able to observe detectable rictor protein levels in cultured smooth muscle cells with available reagents. Nevertheless, the existence of discrete mTOR/raptor and mTOR/riCTOR complexes that are differentially suppressible by rapamycin in smooth muscle cells provides a plausible explanation for the divergent effects of rapamycin on this axis, although it is also possible that rapamycin has mTOR-independent effects that affect AKT activation directly or indirectly.

The paradoxical activation of AKT by rapamycin would suggest that non-mTOR-independent pathways downstream of AKT should also be activated by rapamycin, and indeed that is the case. Forkhead family proteins are known targets of AKT, which phosphorylate and inactivate these transcription factors to allow de-repression of AKT-dependent cellular events. We found that FOXO1 phosphorylation was potently stimulated by rapamycin treatment (Figure 3). This observation is salient in the context of our observations about the diminished migratory effects of rapamycin under high glucose conditions, insofar as unphosphorylated FOXO1 is known to activate p27, which in turn has been shown to suppress smooth muscle cell migration. Phosphorylation of FOXO1 under high-glucose conditions may therefore provide a mechanism for escape from the antimigratory activities of rapamycin. Consistent with this model, rapamycin-dependent suppression of smooth muscle migration is known to be p27-dependent. The ability of rapamycin to reverse ceramide-induced decreases in smooth muscle cell survival (which are known to be dependent on downregulation of AKT) are also generally consistent with rapamycin-induced AKT activation observed in our studies. The observation that rapamycin-dependent survival effects are less dependent on glucose concentrations than are the effects on migration suggests that additional hyperglycemia-dependent cues are necessary to diminish the anti-migratory effects of rapamycin, which in turn would imply that the effects of rapamycin on migration may involve a balance among different signaling pathways.

Taken as a whole, the results of our experiments suggest that the cellular microenvironment can have dramatic and unexpected effects on the action of drugs used clinically in drug-eluting stents. Specifically, a cellular milieu typical of that found in patients with type II diabetes leads to stimulation of potent mitogen activation pathways after treatment with low doses of rapamycin, and results in unintended migration and survival of vascular smooth muscle cells under some circumstances. These observations may have important ramifications in in vivo settings, such as the recent demonstration that suppression of neointimal formation by rapamycin-eluting stents is not sustained beyond 30 days compared with bare-metal stents in a porcine model. This loss of efficacy is caused in part by migration of smooth muscle cells into acellular areas and is associated with decreased p27 expression between 30 and 90 days after stent implantation. Because patients with type II diabetes and other high-risk factors are frequent candidates for DES deployment, and the optimal stent-based treatment for these patients remains a source of controversy, it is important that the biological milieu of this patient population be taken into account when making decisions about therapeutic modalities.

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Comparative Effects of Paclitaxel and Rapamycin on Smooth Muscle Migration and Survival: Role of Akt-Dependent Signaling
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Supplemental Figure Legend

Figure S1. Additional effects of rapamycin and paclitaxel on the AKT signaling axis. A. Western blot analysis was used to detect activation of AKT by measuring the phosphorylation of AKT on residue serine 473 in response to insulin (100 nM) stimulation in smooth muscle cells cultured in low (NG; 5 mM) or high glucose (HG; 25 mM) conditions treated with high-dose paclitaxel (PTXL) at the indicated concentrations for 1 hr prior to insulin stimulation. B,C. Similar studies were performed to detect activation of PDK1 by measuring the phospho-PDK1 activity after insulin stimulation in smooth muscle cells treated with rapamycin (Rapa) or paclitaxel (PTXL) at the indicated concentrations. D. Lysates from the same cells were assayed for PP2A activity using a commercially available kit (Upstate Biotechnology).