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Relative Resistance to Mammalian Target of Rapamycin Inhibition in Vascular Smooth Muscle Cells of Diabetic Donors

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ABSTRACT

Background: Diabetes mellitus is associated with an increased risk of cardiovascular disease. Intimal thickening, a component of cardiovascular disease, entails the proliferation and migration of vascular smooth muscle cells (VSMCs). Inhibition of the mammalian target of rapamycin (mTOR) blocks VSMC proliferation, in part through an increase in the cyclin-dependent kinase inhibitor, $p27^{Kip1}$. The use of mTOR inhibitors, such as rapamycin, is effective clinically in inhibiting intimal thickening. This efficacy is reduced in diabetic subjects, however, suggesting a change in the role of the mTOR pathway in intimal thickening under diabetic conditions.

Methods: To examine whether diabetes induced changes in the role of mTOR in VSMC proliferation, we compared the response to rapamycin of human coronary artery VSMCs from diabetic (DM-huCASMC [human coronary artery smooth muscle cell]) and nondiabetic (ND-huCASMC) subjects.

Results: The DM-huCASMCs exhibited a relative resistance to rapamycin's inhibition of proliferation. Activation of the mTOR effector $p70^{S6kinase}$ was inhibited in rapamycin-treated DM-huCASMCs as in ND-huCASMCs. While ND-huCASMCs exhibited the normal increase in $p27^{Kip1}$ in response to rapamycin treatment, the DM-huCASMCs did not. Additionally, activation of the extracellular signal response kinase pathway was increased in the DM-huCASMCs, suggesting a potential pathway mediating the mTOR-independent decrease in $p27^{Kip1}$.

Conclusion: We conclude that diabetes is accompanied by a relative resistance to the effects of mTOR inhibition on VSMC proliferation through a loss of mTOR's effects on $p27^{Kip1}$ levels. These data provide insight into the effects of insulin resistance on the role of mTOR in regulating intimal thickening.

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INTRODUCTION

Mortality from cardiovascular disease (CVD) is 2 to 4 times higher in diabetic patients than in nondiabetic patients.[1] Multiple aspects of diabetes result in an inflammatory insult to the vasculature, including hyperglycemia,[2] hypoglycemia,[3-5] inflammation,[3,6] and reactive oxygen species.[7-9] While it is clear that this increased injury to the vasculature promotes increased CVD in diabetic patients, changes in the cellular and molecular responses to these insults may also play an important role in increased CVD in the diabetic population.[8,10,11]

One component of the arterial response to injury, intimal hyperplasia, is increased in diabetic patients following percutaneous coronary interventions and leads to increased restenosis.[12,13] Intimal hyperplasia consists largely of vascular smooth muscle cell (VSMC) proliferation and migration. VSMCs isolated both from animal models of diabetes and diabetic patients exhibit increased proliferation and migration, suggesting that VSMCs adopt a prointimal thickening

phenotype in the diabetic setting.[14-16] Intimal thickening also plays a key role in the earliest stages of the pathogenesis of an atherosclerotic lesion when lipid deposition occurs in the extracellular matrix of areas of diffuse intimal thickening.[17-19]

VSMC proliferation and migration are regulated by the cyclin-dependent kinase inhibitor, $p27^{Kip1}$. Quiescent VSMCs maintain elevated levels of $p27^{Kip1}$ that block VSMC proliferation and migration and inhibit neointimal hyperplasia.[20,21] Upon injury, the $p27^{Kip1}$ protein is downregulated through the activation of the mammalian target of rapamycin (mTOR) as neointimal hyperplasia progresses.[21-25] Inhibition of mTOR blocks VSMC proliferation and migration and is an effective strategy in the prevention of in-stent restenosis through the use of drug-eluting stents.[25-28] While drug-eluting stents that deliver mTOR inhibitors are more effective than bare metal stents in diabetic patients, the efficacy of mTOR inhibition is reduced.[29]

Here we report that VSMCs isolated from the coronary arteries of diabetic donors exhibit a relative resistance to the ability of mTOR inhibition to block cell proliferation. Furthermore, we find that the effect of mTOR inhibition on $p27^{Kip1}$ levels is lost in the VSMCs of diabetic donors, suggesting a mechanism for the relative resistance to mTOR inhibition. These data provide a molecular basis for the increased neointimal hyperplasia and the decreased efficacy of mTOR inhibitor–eluting stents in diabetic patients.

METHODS

Cell Culture

Human coronary artery smooth muscle cells (huCASMCs) from diabetic (n=3) and nondiabetic (n=3) donors were obtained from Lonza, Inc. (Walkersville, MD) and maintained in human smooth muscle growth medium (SmGM-2; Lonza) with media changes every 48-72 hours. Rapamycin was obtained from LC Laboratories (Woburn, MA). Cell proliferation assays were performed in triplicate as previously described.[30] Briefly, huCASMCs (2,000) were seeded into 96-well plates and incubated in basal media (SmBM; Lonza) supplemented with 0.5% fetal bovine serum (FBS) overnight. Proliferation was stimulated with SmGM-2 for 72 hours. huCASMCs were used up to passage 6. Data are presented as the mean of the data from the different huCASMC isolates. The half maximal effective concentration (EC_{50}) was calculated using linear regression of the log-transformed mean dose-response data.

Western Blotting

Western blots were prepared as previously described[21] and probed with primary antibodies purchased from BD Biosciences ($p27^{Kip1}$; San Jose, CA), Santa Cruz Biotechnology ($p70^{S6Kinase}$; Santa Cruz, CA), and Cell Signaling Technology (β-actin; Beverly, MA) and with secondary antibodies from Vector Laboratories, Inc. (Burlingame, CA). The $p27^{Kip1}$ and $p70^{S6kinase}$ primary antibodies were used at a 1:1,000 dilution, and the β-actin was used at a 1:2,000 dilution. huCASMCs were serum starved in SmBM supplemented with 0.5% FBS overnight and then incubated in SmGM-2 for 1 hour for the $p70^{S6kinase}$ and overnight for the $p27^{Kip1}$ measurements.

Statistics

All data are expressed as the mean ± standard error of the mean. For comparisons across increasing doses of rapamycin, analysis of covariance was used to test for statistical differences with dose treated as a covariate. $P<0.05$ was considered significant.

RESULTS

huCASMCs Isolated From Diabetic Donors Exhibit a Relative Resistance to Rapamycin

To measure the effect of diabetes on the ability of mTOR inhibition to block VSMC proliferation, we measured the proliferation of huCASMCs isolated from diabetic (DM-huCASMC) and nondiabetic (ND-huCASMC) donors in the presence of increasing doses of the mTOR inhibitor rapamycin (0-100 nM). The DM-huCASMCs exhibited a significant relative resistance to rapamycin treatment compared to ND-huCASMC controls (Figure 1, $P<0.01$). This resistance is seen both as a reduction in the maximal inhibitory effect (69% ± 7% to 38% ± 5%, $P<0.05$) and in a 10-fold shift in the EC_{50} (5-50 nM). These results suggest that diabetes is accompanied by a diminished role for the mTOR pathway in controlling VSMC proliferation.

Regulation of $p27^{Kip1}$ by mTOR Is Reduced in DM-huCASMCs

To test whether the effects of mTOR on its downstream effectors were maintained in the DM-huCASMCs, we measured the ability of rapamycin treatment (0-100 nM) to inhibit the phosphorylation of $p70^{S6kinase}$ and $p27^{Kip1}$ in response to stimulation with growth media. Rapamycin treatment was effective at reducing phosphorylation of $p70^{S6kinase}$ in both the DM-huCASMCs and ND-huCASMCs (Figure 2A), demonstrating that the ability of rapamycin to inhibit mTOR is not lost in the DM-huCASMCs. In contrast, the DM-huCASMCs did not exhibit the increase in $p27^{Kip1}$ protein levels in response to rapamycin treatment seen in the ND-huCASMCs (Figure 2B). Thus, while rapamycin is able to inhibit the mTOR pathway under diabetic conditions, there is a dysregulation of mTOR and $p27^{Kip1}$.

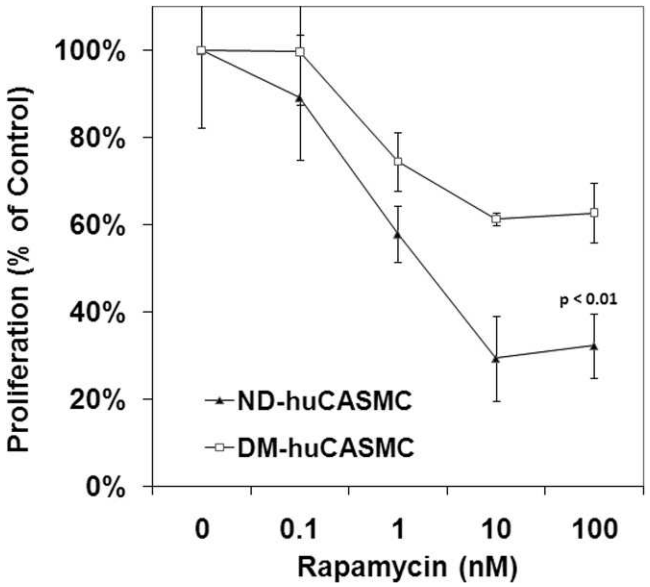

Figure 1. Rapamycin dose-response curves for proliferation of human coronary artery smooth muscle cells (huCASMCs) from nondiabetic (ND-huCASMC) and diabetic (DM-huCASMC) patients.

Dysregulation of $p27^{Kip1}$ and mTOR Is Associated With an Increase in Activation of the Extracellular Signal Response Kinase Pathway by Insulin

Previously, we reported that a similar resistance to mTOR inhibition was associated with increased activation of the extracellular signal response kinases 1/2 (ERK1/2) and decreased Akt activation in response to insulin.[14] We therefore measured phosphorylation of ERK1/2 and Akt at sites promoting activation in response to physiological insulin levels (0-10 pM). The DM-huCASMCs exhibited increased ERK1/2 activation and a loss of Akt activation in response to insulin compared to ND-huCASMCs (Figure 3). Combined with our previous report, these data suggest that the dysregulation of mTOR and $p27^{Kip1}$ seen in the DM-huCASMCs may result from increased activation of the ERK1/2 pathway in response to physiological insulin concentrations.

DISCUSSION

This study reports that huCASMCs isolated from diabetic subjects exhibit a resistance to mTOR inhibition and that this resistance is derived from a dysregulation of mTOR and $p27^{Kip1}$. Initial studies in cultured VSMCs and in the porcine model of vascular injury suggested a critical role for $p27^{Kip1}$ in rapamycin's ability to inhibit neointimal hyperplasia.[21,23-25] Studies in our laboratory mirror the earlier studies that support a role for $p27^{Kip1}$.[14,31] A recent report provided new data in support of a role for $p27^{Kip1}$ in the vascular response to injury in 2 animal models in which S-phase kinase-associated protein (Skp2) was downregulated, increasing $p27^{Kip1}$. Neointimal hyperplasia was reduced both in $Skp2^{-/-}$ mice following carotid ligation and in balloon-injured rat carotids treated with an adenovirus expressing a dominant

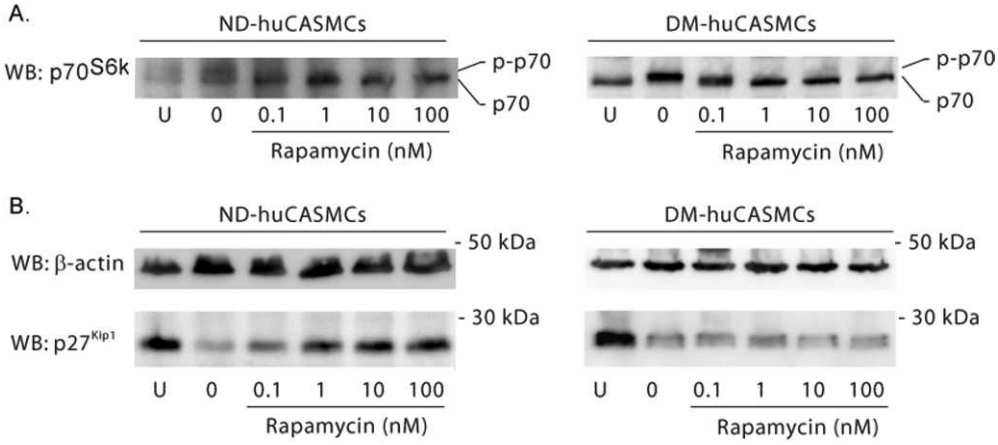

Figure 2. Representative Western blots (WB) of (A) $p70^{S6kinase}$ and (B) $p27^{Kip1}$ in response to incubation with increasing doses of rapamycin. Human coronary artery smooth muscle cells (huCASMCs) in diabetic (DM-huCASMC) and nondiabetic (ND-huCASMC) patients were serum starved overnight (U) and then stimulated with smooth muscle growth medium for 1 hour ($p70^{S6kinase}$) or overnight ($p27^{Kip1}$). Phosphorylated $p70^{S6kinase}$ appears as the slower migrating band labeled p-p70, and unphosphorylated $p70^{S6kinase}$ appears as the faster migrating band labeled p70. β-actin is presented as a loading control for the $p27^{Kip1}$ blots. Note: The lower band in the DM-huCASMC $p27^{Kip1}$ blot is a nonspecific band.

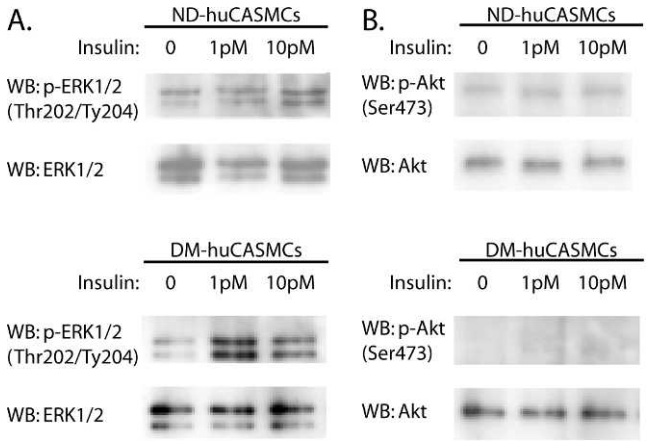

Figure 3. Representative Western blots (WB) of (A) phosporylated and total extracellular signal response kinases 1/2 (ERK1/2) and (B) Akt in response to incubation with increasing doses of insulin. Human coronary artery smooth muscle cells (huCASMCs) were serum starved overnight and then stimulated with smooth muscle basal media supplemented with insulin for 10 minutes. Ser473, serine 473; Thr202, threonine 202; Ty204, tyrosine 204.

negative Skp2$^{-/-}$.[32] Additionally, adenoviral delivery of wild-type Skp2 reduced p27^{Kip1} levels and increased neointimal hyperplasia in minimally injured rat carotids.[32]

Previous reports indicated that VSMCs isolated from animal models of diabetes and from diabetic subjects exhibit increased rates of proliferation.[14-16] Our work builds on those findings by identifying that the regulation of p27^{Kip1} by the mTOR pathway is diminished in the DM-huCASMCs. These findings are similar to those seen in murine BC3H1 cells that were selected for resistance to rapamycin. The rapamycin-resistant BC3H1 cells also exhibited a lack of an increase in p27^{Kip1} in response to rapamycin, while maintaining an intact p70^{S6kinase} response to rapamycin.[24] The mechanism behind the dysregulation of mTOR and p27^{Kip1} warrants further investigation. We recently reported that in VSMCs lacking the insulin receptor, a similar resistance to rapamycin was observed and was linked to an increase in the activation of the ERK1/2 pathway that promoted degradation of p27^{Kip1} messenger RNA (mRNA).[14] We observed a similar increase in ERK1/2 activity in response to insulin in the DM-huCASMCs, suggesting a common mechanism. Because mTOR inhibition blocks degradation of the p27^{Kip1} protein, degradation of p27^{Kip1} mRNA through increased ERK1/2 activity is a potential mechanism for the dysregulation of p27^{Kip1} and mTOR.[14,31] An increase in ERK1/2 activity has also been observed in other animal models of diabetes.[33] Future studies are needed to elucidate the mechanism driving an increase in ERK1/2 activity in the vasculature of diabetic subjects and its impact on the regulation of p27^{Kip1} and intimal thickening.

CONCLUSION

These data demonstrate that huCASMCs isolated from diabetic donors exhibit a relative resistance to the antiproliferative effects of mTOR inhibition. This resistance is derived from a dysregulation of mTOR and p27^{Kip1}, similar to that seen in other rapamycin-resistant cells. Further investigation into the mechanism behind this dysregulation may impact the design of future therapies for vasculoproliferative diseases that target the diabetic population.

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