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Sera from Diabetics does not Alter the Effect of mTOR Inhibition on Smooth Muscle Cell Proliferation

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Abstract

Clinical studies of drug eluting stents delivering the mammalian Target of Rapamycin (mTOR) inhibitor, rapamycin (Sirolimus), have demonstrated a reduced efficacy for these devices in diabetic patients, which suggests the mTOR pathway may cease to be dominant in mediating the vascular response to injury under diabetic conditions. We hypothesized that changes in serum composition accompanying diabetes may reduce the role of mTOR in mediating the vascular response to injury. We measured the ability of a median dose of rapamycin (10 nM) to inhibit the proliferation of human coronary artery smooth muscle cells (huCASMCs) stimulated with serum obtained from diabetic (n = 14) and non-diabetic (n = 16) donors. In an additional analysis, we compared rapamycin's effects on huCASMCs stimulated with the serum of donors with metabolic syndrome (n = 15) versus those without (n = 7). There was no difference in the effect of rapamycin on huCASMC proliferation following stimulation with serum from either donors with diabetes or metabolic syndrome compared to the respective controls. We conclude that the changes in the serum composition common to diabetes and metabolic syndrome are insufficient to diminish the role of mTOR in the progression of cardiovascular disease.

Keywords

Rapamycin; Vascular Smooth Muscle; Diabetes Mellitus; Metabolic Syndrome X; Coronary Restenosis

INTRODUCTION

Drug eluting stents delivering inhibitors of the mammalian Target of Rapamycin (mTOR) are highly effective at minimizing the neointimal hyperplasia that follows percutaneous coronary interventions, confirming the critical role of mTOR in the vascular response to injury 1, 2. mTOR exists as two functionally distinct complexes that together integrate mitogenic stimuli and nutrient sensing to regulate cell growth, proliferation and migration 3–5. Demonstration that the mTOR inhibitor, rapamycin, blocked vascular smooth muscle cell (VSMC) proliferation *in vitro* and neointimal hyperplasia *in vivo* provided the preclinical basis for the development of this class of drugs for use with drug eluting stents^{6, 7}.

Diabetes mellitus is a major risk factor for cardiovascular disease, and the presence of diabetes mellitus diminishes the efficacy of stents eluting mTOR inhibitors^{8,9}. This suggests that under diabetic conditions alternative pathways may emerge as critical mediators of the vascular response to injury diminishing the role of the mTOR pathway. The molecular basis for such a shift could lie in the increase in the serum concentrations of factors with a mitogenic potential toward VSMCs that serve to activate normally dormant signaling pathways that promote VSMC proliferation or to modulate the cellular response to the normal mitogenic signals. At the cellular level, changes in mitogenic signal transduction associated with the development of insulin resistance could also effect which pathways regulate VSMC proliferation¹⁰.

As a first step in better understanding how diabetes alters the role of mTOR in the vascular response to injury, we measured the ability of rapamycin to inhibit proliferation of human coronary artery smooth muscle cells (huCASMCs) stimulated with serum obtained from diabetic and non-diabetic donors. Though metabolic syndrome does not decrease the efficacy of the rapamycin eluting stent in treating coronary artery disease, it does possess many of the characteristics of diabetes mellitus^{11, 12}. For this reason, we also compared the inhibition of huCASMC proliferation by rapamycin following stimulation with serum from donors with and without metabolic syndrome. Both of these analyses demonstrated there was no difference in the ability of mTOR inhibition to block VSMC proliferation, suggesting that the mechanism underlying the decrease in mTOR's role in the vascular response to injury under diabetic conditions involves changes in mitogenic signal transduction that occur in the vasculature.

METHODS

Chemicals and reagents

Rapamycin was purchased from LC Laboratories (Woburn, MA). Human coronary artery basal media (SmBM) and maintenance media (SmGM-2) were obtained from Lonza, Inc. (Walkersville,

Serum Collection

Serum was prepared by centrifugation of whole blood obtained from 30 patients seen in the Cardiac Catheterization Laboratory of Ochsner Medical Center with the approval of the Ochsner Institutional Review Board. Aliquots of each sample were frozen at -80°C until use. Donors were stratified into diabetics and non-diabetics based on a review of their medical history for a diagnosis of diabetes. Donors were also stratified into those with and without metabolic syndrome based on the presence of at least three of the following criteria: diagnosis of type 2 diabetes, diagnosis of hypertension, body mass index (BMI) > 30, uncontrolled high-density lipoprotein (HDL) < 40 mg/dL for men or < 30 mg/dL for women, and uncontrolled triglycerides > 150 mg/dL. Eight of the donors were excluded from the metabolic syndrome analysis because their medical histories lacked an uncontrolled HDL and/or triglyceride measurement. Chronic kidney disease was defined as an estimated glomerular filtration rate < 60 mL/min/1.73 m² calculated using the Modification of Diet in Renal Disease equation¹³.

Tissue Culture and Cell Proliferation Assay

Three separate lots of HuCASMCs obtained from non-diabetic donors were purchased from Lonza and maintained in SmGM-2 with media changes every ~48 hours at 37°C and 5% CO₂. For the cell proliferation assay ~2000 cells were seeded in a 96-well plate and incubated in SmBM supplemented with 0.5% fetal bovine serum (Invitrogen, Inc., Carlsbad, CA) for 24 hours. HuCASMCs were then stimulated with SmGM-2 supplemented with 20% serum obtained from one of the diabetic or non-diabetic donors and either rapamycin (10 nM) or vehicle for ~72 hours. Unstimulated control cells were incubated with fresh SmBM supplemented with 0.5% fetal bovine serum for 72 hours. Cell proliferation was measured

using the CyQuant NF Cell Proliferation Assay (Invitrogen, Inc.) according to manufacturer's instructions. Samples were assayed in duplicate and the assay was performed three times with a different lot of huCASMCs each time. Percent inhibition was calculated as the difference between the vehicle and rapamycin treated samples divided by the vehicle treated sample adjusted for the unstimulated control.

Statistical Analysis

Differences between the percent of inhibition observed in the huCASMCs stimulated with serum from diabetic versus non-diabetic donors were assessed using the Minitab statistical software package (Minitab Inc., State College, PA) and a general linear model treating the individual huCASMC lots as a covariate. Determination of differences in the demographics of the two groups was performed using the two-tailed Fisher's Exact Probability test for the categorical variables and differences in the continuous variables were determined using a two-tailed Student's t-test. A $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Our goal in this study was to assess the ability of serum from donors with diabetes to alter the role of the mTOR pathway in the vascular response to injury. As VSMC proliferation is a critical component of the vascular response to injury and the ability of rapamycin to inhibit it was a primary piece of the preclinical data supporting the development of drug eluting stents, we measured the ability of a median dose of rapamycin to inhibit the proliferation of huCASMCs stimulated with serum collected from 30 donors stratified according to the presence of diabetes. In order to minimize the effects of donors in the non-diabetic group possessing characteristics of early stages of diabetes or metabolic syndrome, we performed a second analysis of this dataset, stratifying according to the presence or absence of metabolic syndrome. Demographics of both the diabetics versus nondiabetics and the metabolic syndrome donors versus control donors were similar with no significant differences observed (Table 1). There was an increase in the number of donors with coronary artery disease, elevation in BMI, decrease in HDL, and increase in 3-Hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase inhibitor (statin) use in the donors with metabolic syndrome as compared to those without. Additionally, none of the donors had chronic kidney disease.

Given that diabetes and metabolic syndrome are major risk factors for cardiovascular diseases and diabetes increases the risk of in-stent restenosis with the drug eluting stents, it might be expected that rapamycin would be less effective at inhibiting the proliferation of huCASMCs stimulated with serum collected from diabetics. In fact the results were virtually identical for rapamycin's anti-proliferative effects in huCASMCs stimulated with serum from either the diabetic or non-diabetic donors as well as from the metabolic syndrome donors versus control (Figure 1). The mean percent inhibition was 53.6% for the non-diabetic serum versus 54.3% for the diabetic serum. In the case of the metabolic syndrome the mean percent inhibition was 51.4% for the metabolic syndrome serum versus 50.7% for control. Comparison of the range and quartiles of the data from these groups further suggests there was no difference in the ability of rapamycin to inhibit proliferation of huCASMCs stimulated with these serum samples. To statistically evaluate the differences in percent inhibition between the two groups, we employed a general linear model that accounted for differences observed between the individual huCASMC lots and obtained a $P = 0.78$ for the diabetic versus non-diabetic serum and $P = 0.68$ for the metabolic syndrome versus control serum. The almost identical nature of the two datasets suggests that the changes in the composition of the serum that accompany diabetes and the metabolic syndrome are insufficient to diminish rapamycin's effects on smooth muscle cell proliferation.

The complexity of both the vascular response to injury, metabolic syndrome, and diabetes makes identifying changes in signal transduction that result in changes in overall efficacy difficult. The goal of this study was to narrow our scope to whether the changes in serum composition that accompany diabetes and metabolic syndrome could alter mTOR's role in the vascular response to injury. Rather than look individually at factors known to fluctuate in response to these conditions (e.g. glucose, insulin, or leptin), we chose to use serum from patients undergoing cardiac catheterization. While this approach limited our knowledge of which pathways were activated, it allowed us to screen all known and unknown components that may affect mTOR's role. It also allowed for antagonistic and synergistic effects to occur.

This study suggests that the molecular mechanisms underlying the effect of diabetes on the role of the mTOR pathway lies, at least in part, in the tissue, however there are limitations that should be noted. As diabetes progresses the serum concentration of many bioactive factors changes. It is possible that as the disease progresses the effects of serum factors on mTOR's role in cardiovascular disease could become more pronounced. The current study is too small to allow for segmentation of the diabetic donor samples, but future studies could address this. Secondly, the serum is collected from patients undergoing cardiac catheterization. This means the population is skewed toward those with vascular disease and the control samples are not necessarily representative of a healthy donor. However, this population may be more representative of the realworld scenario. Finally, direct and indirect effects of the donor's individual therapeutic regimes on the proliferation of the huCASMCs or on rapamycin's potency cannot be ruled out. Both statins and thiazolidinediones, possess anti-proliferative properties toward VSMCs^{14, 15}. To address this, we performed further general linear model regression analyses testing whether the inclusion of samples from donors taking statins (n = 22), thiazolidinediones (n = 3), sulfonylureas (n = 5), metformin (n = 9), repaglinide (n = 1) or insulin (n = 1) altered the response to rapamycin. In all cases the results were insignificant. This suggests that individual donor's therapeutic regime did not alter rapamycin's ability to inhibit huCASMC proliferation; however a larger sample size is required to fully address this issue.

In conclusion, this study demonstrates that rapamycin's ability to inhibit huCASMC proliferation was not altered by stimulation with serum from donors with diabetes or metabolic syndrome. This suggests that changes in the vascular tissue, perhaps combined with changes in serum composition, play a critical role in the diminished effect of rapamycin on restenosis. Future studies examining the effects of insulin resistance on the ability of mTOR inhibitors to inhibit VSMC proliferation are warranted.

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Fig. 1.

Stimulation of huCASMCs with serum from diabetic donors and donors with metabolic syndrome did not alter the ability of rapamycin to inhibit cell proliferation. The proliferation of huCASMCs stimulated with serum from 30 donors in the presence of either rapamycin (10 nM) or vehicle was measured at 72 hours. Percent inhibition by rapamycin was calculated and the data was stratified to compare percent inhibition following stimulation with serum from diabetics to nondiabetics (left panel) and stimulation with serum from donors with metabolic syndrome to those without (right panel). Brackets indicate the range. The boxes indicate the second and third quartiles. The line indicates the median and the ♦ indicates the mean.

Table 1
Comparison of the Demographics and Cardiovascular Risk Factors of the Donors in Each Serum Group

	Non-Diabetic (16)	Diabetic (14)	P- value	Control (7)	Metabolic Syndrome (15)	P- value
Gender						
Male	11 (69%)	9 (64%)	1.000	5 (71%)	10 (67%)	1.000
Female	5 (31%)	5 (36%)		2 (29%)	5 (33%)	
African-American	2 (13%)	3 (19%)	0.640	2 (29%)	2 (29%)	0.560
Smoker	2 (13%)	4 (29%)	0.380	1 (14%)	2 (13%)	1.000
Coronary Artery Disease	8 (50%)	11 (79%)	0.140	2 (29%)	12 (80%)	0.052
Statin Use	11 (69%)	11 (79%)	0.689	3 (43 %)	13 (87%)	0.054
Age (yrs)	60 ± 3	63 ± 2	0.542	61 ± 5	65 ± 2	0.459
Body Mass Index (kg/m2)	28.8 ± 1	29.8 ± 1	0.466	27.6 ± 1	30.7 ± 1	0.050
Cholesterol						
Total (mg/dL)	176 ± 8	160 ± 13	0.319	175 ± 15	156 ± 9	0.312
HDL (mg/dL)	42 ± 2	43 ± 5	0.801	45 ± 4	36 ± 2	0.052
LDL (mg/dL)	109 ± 7	92 ± 9	0.154	107 ± 12	90 ± 7	0.244
Triglycerides (mg/dL)	140 ± 18	171 ± 24	0.316	125 ± 33	190 ± 19	0.118

Data are Presented as Mean ± SEM. HDL, High-Density Lipoprotein; LDL, Low-Density Lipoprotein.