Coronary Interventions

Everolimus-Eluting Stents Improve Vascular Response in a Diabetic Animal Model

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Background—Preclinical evaluation of the vascular response of drug-eluting stents is limited especially in the setting of diabetes mellitus preventing the evaluation of changes in drug-eluting stent design and eluted drugs after clinical use.

Methods and Results—Cultured human aortic endothelial cells were used to assess the differences between sirolimus and its analog, everolimus, in the setting of hyperglycemia on various cellular functions necessary for endothelial recovery. A diabetic rabbit model of iliac artery stenting was used to compare histological and morphometric characteristics of the vascular response to everolimus-eluting, sirolimus-eluting, and bare metal stent placement. Under hyperglycemic conditions, sirolimus impaired human aortic endothelial cell barrier function, migration, and proliferation to a greater degree compared with everolimus. In our in vivo model of diabetes mellitus, endothelialization at 28 days was significantly lower and endothelial integrity was impaired in sirolimus-eluting stent compared with both everolimus-eluting and bare metal stents. Neointimal area, uncovered struts, and fibrin deposition were significantly higher in sirolimus-eluting compared with everolimus-eluting and bare metal stents.

Conclusions—Use of everolimus-eluting stent results in improved vascular response in our preclinical models of diabetes mellitus. (Circ Cardiovasc Interv. 2014;7:526-532.)

Key Words: diabetes mellitus ■ drug-eluting stents

Symptomatic coronary artery disease is a leading cause of morbidity and mortality in patients with diabetes mellitus. Although drug-eluting stents (DES) lower the need for target lesion revascularization in nondiabetic patients, DES use in patients with diabetes mellitus is associated with increased risk for target lesion revascularization (compared with nondiabetic patients) and with increased risk for late stent thrombosis, a phenomenon whose underlying cause is associated with delayed endothelialization.4-10 Such adverse outcomes after coronary revascularization have raised concerns over the efficacy and safety of DES in diabetes mellitus.11,12 The majority of DES used in clinical practice elute sirolimus or its analog (eg, everolimus) that inhibits the mammalian target of rapamycin (mTOR), a serine/threonine kinase via binding of 12-kDa FK506-binding protein (FKBP12), a 12-kDa FK506-binding protein. Key differences between everolimus and sirolimus include increased lipophilicity and decreased binding affinity of everolimus to FKBP12/12.6 compared with sirolimus.13,14 Sirolimus displacement of FKBP12/12.6 from endothelial intracellular calcium release channels can result in endothelial dysfunction through decreased endothelial-dependent relaxation responses via activation of protein kinase C and impaired endothelial barrier function.13,15 This is likely compounded, given the association of diabetes mellitus with increased protein kinase C activation, which is associated with diabetic complications including accelerated atherosclerosis.16 We hypothesize that the use of everolimus may lead to decreased endothelial dysfunction compared with sirolimus, leading to an improved vascular response after DES implantation in the setting of diabetes mellitus. In addition to differences in mTOR inhibition, first-generation sirolimus-eluting stents (SES) compared with newer-generation everolimus-eluting stents (EES) use improved strut construction and more biocompatible polymers in addition to the use of everolimus aimed at both improving arterial healing by facilitating stent endothelialization and suppressing neointimal growth to prevent both restenosis and thrombotic complications.17,18 Clinical trials of newer-generation EES compared with SES in patients with diabetes mellitus are, however, limited in scope and do not clearly differentiate whether these design improvements result in overall clinical benefit.19,20 Preclinical evaluation is clearly needed to define the vascular response of DES in the setting of diabetes mellitus and evaluate improvements in DES design or changes in locally eluted drugs. In this study, we evaluated sirolimus and everolimus using human aortic endothelial cells (HAEC) to compare differences in key cellular processes involved in arterial healing in the setting of hyperglycemia and used a diabetic rabbit model of stenting to define vascular responses to EES, SES, and bare metal stents (BMS).
WHAT IS KNOWN

• First-generation drug-eluting stents are associated with delayed stent endothelialization, especially in patients with diabetes mellitus, and their use is associated with an increased risk of stent thrombosis.
• Everolimus-eluting stents have similar clinical efficacy compared with sirolimus-eluting stents with improved angiographic end points and potentially decreased rates of stent thrombosis in patients with diabetes mellitus.

WHAT THE STUDY ADDS

• In a preclinical model of diabetes mellitus, newer-generation everolimus-eluting stent use is associated with an improved vascular response compared with sirolimus-eluting stents.
• This finding is likely because of differences in the eluted drugs.

Methods

Diabetic Rabbit Model

Twenty-two New Zealand white male rabbits (3–3.5 kg) were made diabetic by means of a single dose of alloxan (100 mg/kg IV). Plasma glucose levels were obtained at baseline before alloxan treatment and every 4 hours for the first 12 hours and every 8 hours for the following 36 hours and daily thereafter. Consistent with previous studies, animals with random plasma glucose consistently >250 mg/dL between 36 hours and daily thereafter were included in the study. Twenty-two New Zealand white male rabbits (3–3.5 kg) were made diabetic by means of a single dose of alloxan (100 mg/kg IV). Plasma glucose levels were obtained at baseline before alloxan treatment and every 4 hours for the first 12 hours and every 8 hours for the following 36 hours and daily thereafter. Consistent with previous studies, animals with random plasma glucose consistently >250 mg/dL between 36 hours and daily thereafter were included in the study. To prevent ketoacidosis, animals with evidence of elevation of blood ketones (monitored daily with reagent strips) were given intramuscular low dose (1–2 U/d) of long-acting insulin (Novolin N) daily. The protocol was approved by the Institutional Animal Care and Committee of Emory University (Atlanta, GA), and all experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. A diagram illustrating the study design is included in Figure I in the Data Supplement.

Rabbit Model of Iliac Artery Stenting, Assessment of Stent Morphometry, Histology, and Endothelialization

New Zealand white adult rabbits underwent endothelial denudation of both iliac arteries using an angioplasty balloon catheter (Maverick, 3.0x12 mm, Boston Scientific, Boston, MA). Subsequently, 3.0x12 mm EES (Xience V, Abbott Vascular, Santa Clara, CA), SES (Cypher, Cordis Corporation, Bridgewater, NJ), or 3.0x12 mm BMS with an identical strut backbone to EES (Multi-link Vision, Abbott Vascular) were deployed at a target stent-to-artery diameter ratio of 1.3:1 in each iliac artery. Each rabbit was randomized for BMS and DES (SES, EES) placement in each iliac artery in a 1:1:1 distribution resulting in an equal number of arterial stenting for each stent type. Stented arteries were harvested at 28 days as previously described. Confocal light microscopy was used to assess stent morphometry and histology. The extent of arterial injury at the site of stent struts was graded by the method of Schwartz et al. En face scanning electron microscopy was used to assess stent endothelialization. See Data Supplement for further details.

Cell Culture and In Vitro Endothelial Function Assays

HAEC (Cell Applications, San Diego, CA) were maintained in endothelial cell growth medium (Cell Applications), and passages 4 through 8 were used for all experiments. For all experiments, endothelial cells were washed once in PBS and before appropriate reagents and media were introduced. Transendothelial electric resistance was measured in real time using electric cell-substrate impedance sensing (ECIS) software (Applied Biophysics, Troy, NJ) and is expressed as specific electric resistance (Ω cm²). Data are presented as the change in resistive portions of the resistance normalized to its value at baseline. Proliferation, apoptosis, and migration assays were performed as previously described. Further experimental details are available in the Data Supplement.

Model of mTOR Inhibition and Hyperglycemia

Sirolimus dose of 500 nmol/L was chosen consistent with our previous work of tissue concentration after SES implantation. The rapamycin analog, everolimus, was chosen at the same concentration to determine its comparative effect on HAEC. Assays were performed under hyperglycemic (30 mmol/L glucose) conditions. Assays were conducted by first exposing HAEC to high glucose for 48 hours followed by sirolimus (500 nmol/L) or everolimus (500 nmol/L) for an additional 24 hours.

Statistical Analysis

Statistical analysis was performed with STATA 9.2 (College Station, TX). All comparisons were performed using linear or Poisson regressions to allow correction for intraclass correlations among groups. In addition, conservative Huber/White/sandwich variance estimates were used, given small sample sizes. All dependent variables were tested for normality with the Wilk–Shapiro test either before or after single-parameter log transformation. All regression computations were bootstrapped with 100 random replications per regression model, justifying the use of parametric estimation methods and correcting for intraclass correlations. Comparisons were made between estimated regression coefficients of each independent variable (ie, $b_{sirol}$ or $b_{everol}$, Tables I–IV in the Data Supplement) or slope/intercept of the regression model. Protection against spuriously significant differences between these 2 regression coefficients was provided by the Bonferroni theorem on a per-table or per-figure basis (with n comparisons each) such that our threshold for statistical significance is $P<α/n$, where $α=0.05$. Further statistical details are available in the Data Supplement.

Results

Endothelial Barrier Function, Cell Proliferation, Migration, and Apoptosis Under Hyperglycemic Conditions

Cultured HAEC were used to assess the effect of sirolimus and everolimus in the setting of hyperglycemia on various cellular functions necessary for endothelial recovery. First, to confirm the effect of our in vitro hyperglycemic model, we conducted immunoblotting for phosphorylated Akt. Attenuation of phosphorylation was seen in the setting of hyperglycemia (30 mmol/L) and insulin stimulation compared with normoglycemia (5 mmol/L), confirming an in vitro model of insulin resistance (Figure II in the Data Supplement).

Using regression modeling comparing the regression coefficient between groups, endothelial barrier function of HAEC treated with sirolimus and everolimus was compared in the setting of hyperglycemia using transendothelial electric resistance and showed a significantly impaired transendothelial electric resistance in hyperglycemia+sirolimus compared with hyperglycemia+everolimus during the measured 24-hour period (Figure 1A). In addition, there was a significant increase in apoptosis with hyperglycemia+everolimus compared with hyperglycemia+sirolimus at 24, 48, and 72 hours (Figure 1B). HAEC proliferation was inhibited to a greater extent in hyperglycemia+sirolimus compared with
hyperglycemia+everolimus at 24 hours (Figure 1C). Migration was significantly decreased by hyperglycemia+sirolimus compared with hyperglycemia+everolimus only in the presence of insulin (Figure 1D).

Diabetes Mellitus Induction and Insulin Signaling

Of the 22 animals that underwent diabetes mellitus induction with alloxan, 6 died from overwhelming hypoglycemia consistent with known mortality from alloxan diabetes mellitus21 and one additional animal did not demonstrate glucose levels >250 mg/dL 1 week after induction and was excluded. All the remaining 15 animals underwent stenting 2 weeks after alloxan induction, resulting in 30 stented arteries (SES, n=10; EES, n=10; and BMS, n=10). All animals underwent stenting without incidence of dissection and thrombosis, and all stents were patent at follow-up angiography. All animals survived the duration of the study without major complications (ie, ketoacidosis). Weights were also not significantly different at the time of diabetes mellitus induction and euthanasia within and between different animal groups (Table 1). Animals received similar daily weight-based doses of insulin both within and between groups. Each group maintained similar average daily blood glucose levels well above 250 mg/dL throughout the study (Table 1). To confirm the validity of our animal model, mitogenic components of the insulin signaling pathway, Akt and extracellular signal-regulated kinases, were examined by immunoblotting in whole iliac arteries of our diabetic model.

Table 1. Diabetic Rabbit Average Weight at Alloxan Induction and Euthanasia and Average Daily Blood Glucose Levels After Induction

<table>
<thead>
<tr>
<th></th>
<th>BMS (n=10)</th>
<th>EES (n=10)</th>
<th>SES (n=10)</th>
<th>p Value (*b&lt;sub&gt;EES&lt;/sub&gt;=b&lt;sub&gt;SES&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg) at induction</td>
<td>3.3±0.1</td>
<td>3.1±0.1</td>
<td>3.2±0.2</td>
<td>0.11</td>
</tr>
<tr>
<td>Weight (kg) at euthanasia</td>
<td>3.1±0.2</td>
<td>3.2±0.3</td>
<td>3.5±0.5</td>
<td>0.62</td>
</tr>
<tr>
<td>Glucose, mg/dL*</td>
<td>393±34</td>
<td>351±78</td>
<td>331±51</td>
<td>0.91</td>
</tr>
</tbody>
</table>

*indicates the linear regression coefficient for each group; BMS, bare metal stents; EES, everolimus-eluting stents; and SES, sirolimus-eluting stents.

*P value computed after normalizing log transformation. Bonferroni threshold of significance is 0.0167.

Figure 1. Endothelial barrier function, proliferation, apoptosis, and migration in human aortic endothelial cells (HAEC) in hyperglycemic Environments. HAEC were incubated in hyperglycemic (30 mmol/L) environment for 72 hours with treatment with everolimus [hyperglycemia (HG)+everolimus [EVL]] or sirolimus (HG+sirolimus [SRL]) for 24 hours. A, HAEC endothelial barrier function was assessed by transendothelial electric resistance (TEER) showing significantly impaired barrier function with HG+SRL compared with HG+EVL when analyzed with linear regression modeling (n=3 wells; P=0.0006; Bonferroni threshold=0.025). B, HAEC undergoing late/definite apoptosis were measured by flow cytometry (propidium iodide and fluorescein isothiocyanate [FITC]-Annexin–positive stained cells) shown quantitatively with examples of bivariate plots at 24 hours shown to the left. Apoptosis in HG+EVL was significantly higher at 24, 48, and 72 hours compared with HG+SRL when analyzed with Poisson regression (n=3; P<0.0005; Bonferroni threshold=0.017). C, HAEC proliferation was measured by bromodeoxyuridine (BrdU) incorporation and was significantly decreased by HG+SRL compared with HG+EVL (n=4; linear regression, P<0.0005; Bonferroni threshold=0.017). D, HAEC migration was measured by modified Boyden chamber assays and expressed as percent cells migrated compared with HG. In the presence of insulin (ins), migration was significantly decreased by HG+SRL compared with HG+EVL (n=4; linear regression, P<0.0005; Bonferroni threshold=0.0083) however not in the absence of insulin (P=0.0125).
2 weeks after alloxan induction. We observed attenuation of Akt (Ser 473) phosphorylation in our diabetic model compared with control animals, with extracellular signal-regulated kinases (Thr202/Tyr204) phosphorylation remaining preserved (Figure II in the Data Supplement) recapitulating molecular features of insulin resistance in the vasculature.

Morphometric Analysis

In 28-day stents, neointimal area was significantly lower in EES compared with both SES and BMS; however, this difference did not persist when normalized to injury score (Table 2; Figure 2A). Internal and external elastic lamina and medial area were not significantly different between groups (Table 2). Percent stenosis was significantly higher in BMS compared with EES and SES, however, without significant difference between EES and SES (Table 2).

Histology

The percentage of uncovered struts and struts surrounded by fibrin was significantly higher in SES compared with BMS or EES (Table 3). No differences were seen in the number of inflammatory cells and percentage of giant cells between groups (Table 3).

Scanning Electron Microscopy

En face scanning electron microscopy of 28-day stented segments demonstrated near-complete endothelial coverage in arteries implanted with BMS and EES, whereas SES demonstrated significantly decreased coverage over struts (Figure 3A and 3B). Arteries with SES consistently also showed disrupted endothelial cell junctions compared with BMS and EES (Figure 3A, right).

Discussion

This study is the first to compare the vascular response between different –limus-based DES in a diabetic animal model of stenting. In this study, 28-day EES were compared with SES and BMS with an identical strut backbone to EES after placement in the iliac arteries of insulin-dependent diabetic rabbits. Alloxan induction of diabetes mellitus achieved a significant degree of hyperglycemia in our animal model throughout the study period requiring the use of long-acting insulin (1–2 U of Novolin N) during this period (Table 1) and recapitulated molecular features of insulin resistance (Figure II in the Data Supplement). In this model, EES displayed an improved vascular response compared with SES with near-complete endothelialization/covered strut and less fibrin deposition. In addition, robust endothelial barrier integrity comparable with BMS with similar antirestenosis efficacy to SES was observed. In regard to key cellular functions for endothelialization, everolimus demonstrated improved endothelial barrier function, proliferation, and migration compared with sirolimus while conversely having increased apoptosis, all under hyperglycemic conditions. Everolimus is a 40-O-hydroxyethyl derivative of sirolimus that similarly inhibits mTOR through binding to FKBP12/12.6, a ubiquitous 12-kDa FK506-binding protein. Although everolimus displays similar inhibition of mTOR compared with sirolimus in vivo, in vitro assays previously showed decreased affinity of everolimus to FKBP12/12.6. In this study, we observe poor endothelial barrier function with respect to hyperglycemia+sirolimus compared with

Table 2. Morphometric Characteristics of Stents Implanted in a Diabetic Animal Model at 28 Days

<table>
<thead>
<tr>
<th></th>
<th>BMS (n = 7)</th>
<th>EES (n = 7)</th>
<th>SES (n = 7)</th>
<th>P Value (bEES=bSES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEL area, mm²</td>
<td>5.99±0.54</td>
<td>5.70±0.45</td>
<td>6.22±0.56</td>
<td>0.044</td>
</tr>
<tr>
<td>EEL area, mm²</td>
<td>6.29±0.52</td>
<td>5.93±0.51</td>
<td>6.51±0.60</td>
<td>0.034</td>
</tr>
<tr>
<td>Medial area, mm²</td>
<td>0.30±0.07</td>
<td>0.23±0.08</td>
<td>0.23±0.04</td>
<td>0.066</td>
</tr>
<tr>
<td>Neointimal area, mm²</td>
<td>1.08±0.20</td>
<td>0.60±0.09</td>
<td>0.84±0.18</td>
<td>0.004*</td>
</tr>
<tr>
<td>Stenosis, %</td>
<td>18.1±3.3</td>
<td>10.6±1.8</td>
<td>13.4±2.5</td>
<td>0.016</td>
</tr>
<tr>
<td>Injury score†</td>
<td>0.30±0.17</td>
<td>0.06±0.06</td>
<td>0.13±0.08</td>
<td>0.234</td>
</tr>
<tr>
<td>Neointimal area/injury score†</td>
<td>8.48±1.57</td>
<td>4.72±1.45</td>
<td>6.58±1.45</td>
<td>0.717</td>
</tr>
</tbody>
</table>

b indicates the linear regression coefficient for each group; BMS, bare metal stents; EEL, external elastic lamina; EES, everolimus-eluting stents; IEL, internal elastic lamina; and SES, sirolimus-eluting stents.

*P value < Bonferroni threshold of significance 0.0071.
†P value computed after normalizing log transformation.

Figure 2. Morphometric and histological examples of 28-day diabetic rabbit iliac artery stents. Low- (A) and high-power magnification photomicrographs (B) of diabetic rabbit iliac arteries implanted with bare metal stents (BMS), everolimus-eluting stents (EES), and sirolimus-eluting stents (SES) at 28 days. Detailed morphometric and histological examination showed significantly increased neointima area, percent uncovered struts, and struts with fibrin with SES compared with EES (Table 2).
hyperglycemia+everolimus in the setting of hyperglycemia at 24 hours (Figure 1A) in vitro and correspondingly poor endothelial junctional integrity seen in vivo with scanning electron microscopic analysis of SES compared with EES and BMS (Figure 3A, right). Although displacement of FKBP12/12.6 by sirolimus from endothelial intracellular calcium release channels can result in increased intracellular calcium leading to endothelial dysfunction through calcium-dependent phosphorylation of protein kinase C,13 this suggests that decreased binding affinity of everolimus to FKBP12/12.6 may lead to improved endothelial function after delivery from DES. We have recently conducted a study showing that sirolimus disrupts vascular endothelial cadherin homeostasis, leading to impaired endothelial barrier function likely through displacement of FKBP12/12.6 and increased intracellular calcium rather than mTOR inhibition.15 This mechanism likely works in synergy with protein kinase C phosphorylation seen in diabetes mellitus to impair endothelial function that has also been observed in diabetic animal models.22 This study suggests that the less avid FKBP12/12.6 binding with everolimus compared with sirolimus may result in improved endothelial barrier function and integrity, explaining the differential vascular response observed between the 2 drugs.

With respect to proliferation and apoptosis, everolimus had higher apoptosis, especially initially at 24 hours, compared with sirolimus (Figure 1B). However, inhibition of HAEC proliferation and migration was greater with sirolimus compared with everolimus treatment. This suggests that sirolimus may also differentially inhibit Akt compared with everolimus because Akt activity is involved in HAEC migration and proliferation.24,30 Overall, our findings suggest that although the efficacy of EES and SES was similar, there were significant differences in endothelialization and endothelial integrity seen in vivo and endothelial barrier function, proliferation, and migration in vitro, suggesting that EES/everolimus may improve vascular endothelial response in diabetic environment.

Preclinical Findings
Previous studies in a nondiabetic rabbit model showed similar improvement in endothelialization with comparator DES using newer strut constructs (ie, EES); however, these improvements did not persist past 14 days.22 This differs from our diabetic model in which differences were seen at 28 days between EES and SES. In addition, there was a lesser degree of endothelialization seen with thicker strut SES in our diabetic model (37.7±1.4%) versus historical nondiabetic rabbit model (>60%) with preservation of endothelialization in thinner strut EES in both models.22 In this study, morphometric and histological characteristics are more favorable for EES with significant reductions in neointimal area and improvement in the percentage of uncovered struts and struts with fibrin compared with SES persisting ≤28 days. Although this can be partly explained with lower injury scores of thinner constructed EES compared with SES (Figure 4), similar constructed BMS do not maintain these advantages, suggesting that this is likely because of components beyond strut construction such as the durable polymer used or eluted drug. There was an observed numeric, but not significant, increase in inflammatory cells with SES compared with EES consistent with the more rapid neointimal area response between EES and SES. In addition, there was a lesser degree of endothelial coverage with SES compared with EES consistent with an increased inflammatory response, which is likely as a result of polymer biocompatibility because everolimus and sirolimus have equivalent immunosuppressant activity (Figure 4).4,22,31 This suggests that the improvement of endothelialization/percentage of uncovered struts and endothelial barrier

<table>
<thead>
<tr>
<th></th>
<th>BMS</th>
<th>EES</th>
<th>SES</th>
<th>𝑃 Value (Bonferroni threshold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% struts with fibrin*</td>
<td>6.8±3.4</td>
<td>12.8±11.1</td>
<td>27.3±15.9</td>
<td>0.008†</td>
</tr>
<tr>
<td>% uncovered struts*</td>
<td>14.6±28.1</td>
<td>35.1±30.8</td>
<td>67.2±24.6</td>
<td>0.005†</td>
</tr>
<tr>
<td>% struts with giant cells</td>
<td>45.6±24.0</td>
<td>47.8±16.6</td>
<td>52.4±22.4</td>
<td>0.65</td>
</tr>
<tr>
<td>Inflammatory cells*</td>
<td>24.3±30.4</td>
<td>50.0±39.2</td>
<td>65.0±66.6</td>
<td>0.68</td>
</tr>
</tbody>
</table>

*P value computed after normalizing log transformation.†P value < Bonferroni threshold of significance (0.0125).
function in newer construction EES are because of the differences in its mTOR inhibition compared with SES (Figure 4).

Clinical Implications
Although DES represent a major advance in the treatment of symptomatic coronary artery disease, their use in patients with diabetes mellitus continues to be associated with poorer outcomes after coronary revascularization.\(^\text{35}\) Diabetes mellitus patients treated with both SES and EES have higher repeat revascularization rates than nondiabetic individuals, and diabetes mellitus is an independent risk factor for late stent thrombosis. Our data suggest that both differences in mTOR inhibition and improvements in strut construction in EES improve vascular healing responses compared with SES while preserving antirestenotic efficacy. Dedicated clinical trials comparing responses between these 2 DES in patients with diabetes mellitus are generally lacking. The Everolimus-Eluting Stent Versus Sirolimus-Eluting Stent Implantation for De Novo Coronary Artery Disease in Patients With Diabetes Mellitus (ESSENCE-DIABETES) study and subgroup analysis of both the Efficacy of Xience/Promus Versus Cypher to Reduce Late Loss After Stenting (EXCELLENT) and Intracoronary Stenting and Angiographic Results: Test Efficacy of 3 Limus-Eluting Stents (ISAR-TEST)-4 studies compared SES and EES in patients with diabetes mellitus and found improved angiographic findings with EES compared with SES.\(^\text{9,20,33}\) Although the ISAR-TEST-4 diabetic subgroup showed a trend toward improved thrombotic complications with EES, these studies overall were not powered to examine late stent thrombosis that might be expected to improve with EES. In addition, impaired endothelial barrier function may lead to accelerated neointimal atherosclerosis (neointimal hyperplasia) in DES because of various mechanisms, one of which includes –limus-based mTOR inhibition.\(^\text{15}\) Although little data exist on neointimal hyperplasia in diabetic patients undergoing DES placement, this study suggests that neointimal hyperplasia is likely augmented in diabetes mellitus.\(^\text{16,34}\) Although durable polymer SES are no longer clinically available, renewed sirolimus use in the setting of bioabsorbable DES may further emphasize these differences in mTOR inhibition.\(^\text{15}\) Overall, our data suggest that changes in both mTOR inhibition and stent design can be evaluated in a preclinical diabetic animal model of stenting, which interestingly shows improved vascular responses because of the type of mTOR inhibition in addition to improvement in stent design.

Limitations
Although our animal model of arterial stenting provides key insights into the vascular response under diabetic conditions, it lacks significant comorbid conditions such as atherosclerosis. This in turn may underestimate the degree of impaired endothelialization and suggests that the observed improvement in vascular response with everolimus might be more pronounced.\(^\text{16}\) Another limitation was our inability to measure mTOR activity in the stented arteries. The mTOR complex is upstream from canonical mTOR effectors (ie, Akt) in addition to those outside the canonical mTOR pathway (ie, FKBP12.6).\(^\text{15}\) By measuring mTOR activity, this would allow an indirect measure of –limus efficacy on mTOR-related pathways known to effect vascular endothelialization.\(^\text{15}\)

Conclusions
This preclinical study, using a diabetic animal model of arterial stenting, suggests that there is an improved vascular response with newer-generation DES, which is interestingly because of the type of local mTOR inhibition.

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Disclosures
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References


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Supplemental Material

Supplemental Methods

Stent Procedure. Two weeks after alloxan treatment, anesthetized adult male New Zealand White rabbits underwent endothelial denudation of both iliac arteries using an angioplasty balloon catheter (Maverick, 3.0 × 12 mm, Boston Scientific, Boston, MA). Subsequently, 3.0 x 12 mm everolimus-eluting stent (EES) (Xience V, Abbott Vascular, Santa Clara, CA), sirolimus-eluting stent (SES) (Cypher, Cordis Corporation, Bridgewater, NJ) or 3.0 x 12 mm bare metal stent with an identical strut backbone to EES (BMS) (Multi-link Vision, Abbott Vascular) were deployed at a target stent-to-artery diameter ratio of 1.3:1 in each iliac artery, respectively. All stents were successfully deployed in our animal model without incidence of dissection or thrombosis. Each rabbit was randomized for BMS and DES (SES, EES) placement in each iliac artery in a 1:1:1 distribution resulting in an equal number of arterial stenting for each stent type. All animals remained healthy for the duration of the 28 day experiment prior to harvest.

Antithrombotic Regimen. All animals received aspirin (40 mg/day, orally) 24-hours before catheterization with continued dosing throughout the study. A single dose of intra-arterial heparin (150 IU/kg) was administered at the time of catheterization.

Stent Harvest. Stents were harvested at 28 days after implantation. Animals were re-anesthetized and follow-up angiography was performed to verify patency. Euthanasia was performed subsequently with an overdose of Beuthanasia-D given intravenously. Stented arteries were perfusion fixed in situ with 4% neutral paraformaldehyde after perfusion with Ringer’s Lactate. The samples were bisected longitudinally and processed for en face scanning electron microscopy (SEM) or light microscopy as previously described.¹
**Stent analysis.** All arterial segments were examined blindly. Computerized planimetry was performed on all stented arterial sections, as previously described.\(^1,2\) Percent luminal stenosis was calculated with the following formula: neointimal area divided by the external elastic lamina times 100. Fibrin deposition, the number of giant cells around stent struts, and number of inflammatory cells were quantified and expressed as the percent of struts surrounded by fibrin, the percent of struts surrounded by giant cells, and the total number of inflammatory lining the lumen of the vessel, respectively. SEM was quantified as previously described.\(^3\)

**Transendothelial electrical resistance.** HAECs were seeded on a gelatin-coated gold electrode (5.0×10\(^4\) cells/cm\(^2\)) and grown to confluence. The small electrode and the larger counter-electrode were connected to a phase-sensitive lock-in amplifier. A constant current of 1 µA was supplied by a 1-V, 4000-Hz AC signal connected serially to a 1-MΩ resistor between the small electrode and larger counter-electrode. The voltage was monitored by a lock-in amplifier, stored, and processed by a personal computer. The same computer controlled the amplifier output and switched the measurement to different electrodes in the course of an experiment. Before each experiment, endothelial monolayers were washed with serum-free growth medium and used for measuring changes in TEER. TEER was measured in real-time using ECIS software (Applied Biophysics, Troy, NJ) and is expressed as specific electrical resistance (\(\Omega \text{ cm}^2\)). Data are presented as the change in resistive portions of the resistance normalized to its value at time 0.\(^4\)

**Proliferation Assay.** After experimental conditions were applied in endothelial cell growth medium (Cell Applications), cell proliferation was quantified by the BrdU Cell
Proliferation Assay (Merck KGaA, Darmstadt, Germany) at 24 hours as previously described. This assay was conducted in 96 well culture plates and proliferation was quantified by measuring absorbance at dual wavelengths of 450-540 nm. Results were normalized to HAEC controls. To visualize the amount of BrdU uptake, HAECs underwent staining with Diaminobenzidine (DAB) against the HRP-linked mouse anti-goat secondary antibody to the primary BrdU antibody used in the proliferation assay. Hematoxylin was used as a background counterstain.

**Cell Migration Assay.** After experimental conditions were applied in endothelial cell growth medium (Cell Applications), cell migration assays were performed using modified Boyden chambers (6.5-mm polycarbonate transwell filter inserts with 8 mm pores, Corning, NY). HAEC were trypsinized, centrifuged, and resuspended in basal serum media supplemented with 0.5% calf serum (Cell Application). HAEC were treated or without 100 nmol/L insulin for 1 hour and $10^5$ cells/ml in 300 µL of low serum media were seeded onto the top of the Transwell filters and the bottom wells were filled with 700 µL of low serum media. Cells were incubated at 37°C for 4 hours. Cells migrated to the bottom wells were stained with 1 µl calcein and the number of green cells was scored for at least 4 fields (10 x objective) per filter under florescent microscopy. The average number of cells that migrated per filter was calculated and normalized to control.

**Apoptosis Assay.** After experimental conditions were applied in endothelial cell basal medium (Cell Applications), HAEC were trypsinized, centrifuged, and resuspended at a concentration of $10^6$ cells/ml in binding buffer (BD Bioscience, San Jose, CA). 100 µl of the suspension was stained with 5 µl Annexin-FITC and 5 µl Propidium Iodide (BD
Bioscience, San Jose, CA) and was incubated for 15 minutes at room temperature and an additional 400 µl of binding buffer was added. In addition a positive control was constructed from heat shocked HAEC and similarly stained. Flow cytometry was used to analyze cells stained positive for Annexin-FITC and Propidium Iodide indicating cells undergoing late/definite apoptosis.

**Immunoblotting.** Protein from rabbit iliac arteries 14 days post alloxan induction and cultured human aortic endothelial cells (HAECs) was processed and separated on polyacrylamide gel as previously reported.\(^6\) Blot membranes were incubated with commercially available antibodies against phospho-Akt (Ser 473), Akt, ERK and phosphos-ERK (Thr202/Tyr204) (Cell Signaling, Danvers, MA). Reactive bands were detected by chemiluminesence and quantified using area x density analysis with Quantity One 4.5.2 1-D Analysis Software (Bio-Rad, Hercules, CA).

**Statistical Methods**

All hypothesis tests were performed with STATA 9.2 statistical software (College Park, TX). In order to apply methods that adequately account for the instability of small sample variance estimates, and to correct for intra-class correlations among clustered dependent variables (due to multiple observations per rabbit or per HAEC sample), we conducted all comparisons of everolimus vs sirolimus results using linear or Poisson regressions. Everolimus and Sirolimus were coded as binary independent variables (0,1) and were entered in the regressions as “predictor” variables. This strategy allowed us to use the ‘robust’ and ‘cluster’ options available in the STATA ‘regress’ command to calculate conservative Huber/White/Sandwich variance estimates and to correct for intra-class correlations.
All dependent variables were tested for validity of the assumption of Gaussian errors (except for count data that were analyzed with Poisson regression) with the Wilk-Shapiro test. When this test failed, the dependent variable was transformed by the single-parameter log transformation: \( y' = \log(y-k) \), using the STATA Inskew0 command, where the parameter, \( k \), was estimated from all the data without regard to subgroup. When the \( y' \) data were retested with Wilk-Shapiro they were all compatible with assumed Gaussian error distributions, with the exception of the TEER regression residuals, which were adequately de-skewed by Inskew0 but were still not Normal. But since the TEER vs. time regression was highly linear, and linear regression is generally robust with respect to non-Normal residuals when the underlying model is linear, this non-Normality was regarded as unlikely to impair the findings for the TEER datasets.

At the statistical reviewer’s useful suggestion, we ran all regression computations as bootstrapped runs with 100 random replications per regression model. This helps to justify our use of parametric estimation methods, which, in addition to having greater flexibility and power than non-parametric methods, allow an available calculation that corrects for intra-class correlations.

Most comparisons in tables 1-3 and Figures 1 and 3 were comparisons of the estimated regression coefficients of the binary independent variables EES and SES. These coefficients are labeled ‘\( b \)’ in the cells of the tables. The relevant linear model is: \( y = c + \beta_{ees} \times EES + \beta_{ses} \times SES + \epsilon \), where \( c \) is a common constant and \( \epsilon \) is a common Gaussian error-distribution. Estimates of the regression coefficients \( \beta_{ees} \) and \( \beta_{ses} \) are represented in the tables as \( b_{ees} \) and \( b_{ses} \). The comparisons that test the \( H_0 \) hypothesis, \( b_{ees} = b_{ses} \), were performed with the use of the STATA 9.2 ‘lincom’ post-estimation command after
each linear regression. Similar comparisons were made with baseline (i.e. BMS or HG) and denoted as b(0). P-values for the respective comparisons were either denoted as p(b(0)) for comparison with baseline or p(b_{EES} = b_{SES}) for comparisons between independent groups. Protection against spuriously significant differences between these two regression coefficients was provided by the Bonferroni Theorem on a per-table or per-figure basis. That is, if a given table has n significance tests, then we adjust our nominal threshold for statistical significance, \( \alpha = 0.05 \) to the requirement that the p-value estimated by the ‘regress’ program in STATA be \( p < \alpha/n \). Regression coefficients shown below (supplemental table 1-3). Power analysis for both in vivo and in vitro studies was based on previous studies using these animal and molecular models.\(^7,8\)

**Supplemental table 1**

<table>
<thead>
<tr>
<th></th>
<th>BMS (n = 10)</th>
<th>EES (n = 10)</th>
<th>SES (n = 10)</th>
<th>p(b_{EES}=b_{SES})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg) at</td>
<td>3.3 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>3.2 ± 0.2</td>
<td>0.11</td>
</tr>
</tbody>
</table>
| Induction        | b = -0.22 ± 0.07  
\( p(b=0)= 0.001 \)  | b = -0.09 ± 0.06  
\( p(b=0)= 0.13 \)  |               |
| Weight (kg) at   | 3.1 ± 0.2    | 3.2 ± 0.3    | 3.5 ± 0.5    | 0.62              |
| Sacrifice        | b = -0.014±0.13  
\( p(b=0)= 0.91 \)  | b = 0.066 ± 0.14  
\( p(b=0)= 0.64 \)  |               |
| Glucose (mg/dl)  | 393 ± 34     | 351 ± 78     | 331 ± 51     | 0.91              |
| **              |             |              |              |                   |
\( ** \) p-value computed after normalizing Log transformation. b is the linear regression coefficient for each group with b(0) representing baseline (i.e. BMS). Bonferroni threshold of significance is 0.0167.
### Supplemental table 2

<table>
<thead>
<tr>
<th></th>
<th>BMS (n = 7)</th>
<th>EES (n = 7)</th>
<th>SES (n = 7)</th>
<th>p(b&lt;sub&gt;EES&lt;/sub&gt;=b&lt;sub&gt;SES&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEL area, mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5.99 ± 0.54</td>
<td>5.70 ± 0.45</td>
<td>6.22 ± 0.56</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>b = -0.29±0.26</td>
<td>p(b=0)= 0.26</td>
<td>b = 0.23±0.31</td>
<td>p(b=0)= 0.45</td>
</tr>
<tr>
<td>EEL area, mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>6.29 ± 0.52</td>
<td>5.93 ± 0.51</td>
<td>6.51 ± 0.60</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td>b = -0.36±0.29</td>
<td>p(b=0)= 0.20</td>
<td>b = 0.22±0.27</td>
<td>p(b=0)= 0.41</td>
</tr>
<tr>
<td>Medial area, mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.30 ± 0.07</td>
<td>0.23 ± 0.08</td>
<td>0.23 ± 0.04</td>
<td>0.066</td>
</tr>
<tr>
<td></td>
<td>b = -0.071 ± 0.043</td>
<td>p(b=0)= 0.10</td>
<td>b = -0.016 ± 0.029</td>
<td>p(b=0)= 0.58</td>
</tr>
<tr>
<td>Neointimal area, mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.08 ± 0.20</td>
<td>0.60 ± 0.09</td>
<td>0.84 ± 0.18</td>
<td>0.004*</td>
</tr>
<tr>
<td></td>
<td>b = -0.48 ± 0.078</td>
<td>p(b=0)&lt; 0.0005*</td>
<td>b = -0.24 ± 0.078</td>
<td>p(b=0)= 0.002*</td>
</tr>
<tr>
<td>Stenosis, %</td>
<td>18.1 ± 3.3</td>
<td>10.6 ± 1.8</td>
<td>13.4 ± 2.5</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>b = -7.50 ± 1.25</td>
<td>p(b=0)&lt; 0.0005*</td>
<td>b = -4.69 ± 1.3</td>
<td>p(b=0)&lt; 0.0005*</td>
</tr>
<tr>
<td>Injury score **</td>
<td>0.30 ± 0.17</td>
<td>0.06 ± 0.06</td>
<td>0.13 ± 0.08</td>
<td>0.234</td>
</tr>
<tr>
<td></td>
<td>b = -0.24 ± 0.065</td>
<td>p(b=0)&lt; 0.0005</td>
<td>b = -0.17 ± 0.077</td>
<td>p(b=0)= 0.01</td>
</tr>
<tr>
<td>Neointimal area/Injury score **</td>
<td>8.48 ± 1.57</td>
<td>4.72 ± 1.45</td>
<td>6.58 ± 1.45</td>
<td>0.717</td>
</tr>
<tr>
<td></td>
<td>b = 4.91 ± 3.59</td>
<td>p(b=0)= 0.082</td>
<td>b = 3.66 ± 3.14</td>
<td>p(b=0)= 0.22</td>
</tr>
</tbody>
</table>

* p-value < Bonferroni threshold of significance 0.0071. ** p-value computed after normalizing log transformation. . b is the linear regression coefficient for each group with b(0) representing baseline (i.e. BMS). IEL – internal elastic lamina, EEL – external elastic lamina.
### Supplemental table 3

<table>
<thead>
<tr>
<th></th>
<th>BMS (n = 7)</th>
<th>EES (n = 7)</th>
<th>SES (n = 7)</th>
<th>p(b&lt;sub&gt;EES&lt;/sub&gt;=b&lt;sub&gt;SES&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% struts with fibrin</td>
<td>6.8 ± 3.4</td>
<td>12.8 ± 11.1</td>
<td>27.3 ± 15.9</td>
<td>0.008*</td>
</tr>
<tr>
<td>**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>b = 6.07 ± 4.32</td>
<td>p(b=0)= 0.067</td>
<td>b = 20.6 ± 5.33</td>
<td>p(b=0)&lt;0.0005*</td>
</tr>
<tr>
<td>% uncovered struts</td>
<td>14.6 ± 28.1</td>
<td>35.1 ± 30.8</td>
<td>67.2 ± 24.6</td>
<td>0.005*</td>
</tr>
<tr>
<td>**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>b = 20.4 ± 16.0</td>
<td>p(b=0)= 0.13</td>
<td>b = 52.5 ± 14.8</td>
<td>p(b=0)&lt; 0.0005*</td>
</tr>
<tr>
<td>% struts with giant cells</td>
<td>45.6 ± 24.0</td>
<td>47.8 ± 16.6</td>
<td>52.4 ± 22.4</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>b = 2.22 ± 11</td>
<td>p(b=0)= 0.84</td>
<td>b = 6.83 ± 9.9</td>
<td>p(b=0)= 0.49</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>24.3 ± 30.4</td>
<td>50.0 ± 39.2</td>
<td>65.0 ± 66.6</td>
<td>0.68</td>
</tr>
<tr>
<td>**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>b = 25.8 ± 18</td>
<td>p(b=0)= 0.28</td>
<td>b = 40.7 ± 26</td>
<td>p(b=0)= 0.10</td>
</tr>
</tbody>
</table>

* p-value < Bonferroni threshold of significance (0.0125). ** p-value computed after normalizing log transformation. b is the linear regression coefficient for each group with b(0) representing baseline (i.e. BMS).

### Supplemental table 4

<table>
<thead>
<tr>
<th></th>
<th>BMS (n = 7)</th>
<th>EES (n = 7)</th>
<th>SES (n = 7)</th>
<th>p(b&lt;sub&gt;EES&lt;/sub&gt;=b&lt;sub&gt;SES&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent Endothelialization</td>
<td>99.2 ± 2.0</td>
<td>89.2 ± 6.6</td>
<td>31.2 ± 5.2</td>
<td>&lt; 0.0005*</td>
</tr>
<tr>
<td></td>
<td>b = -5±3.83</td>
<td>p(b=0)= 0.145</td>
<td>b = -61.7±9.12</td>
<td>p(b=0)&lt; 0.0005*</td>
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</tbody>
</table>

* p-value < Bonferroni threshold of significance (0.017). b is the linear regression coefficient for each group with b(0) representing baseline (i.e. BMS).
Supplemental Figure 1

- Phase I – Diabetes Induction
- Phase II – DES Evaluation

Animals arrive → Alloxan Injection → Harvest for Light Microscopy/SEM

Day 0 → Day 7 → Day 14 → Day 21 → Day 49

Stent Placement

daily blood glucose to evaluate success of diabetes induction protocol
Supplemental Figure 2

A

<table>
<thead>
<tr>
<th>No-DM</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAkt</td>
<td></td>
</tr>
<tr>
<td>Akt</td>
<td></td>
</tr>
<tr>
<td>pErk</td>
<td></td>
</tr>
<tr>
<td>Erk</td>
<td></td>
</tr>
</tbody>
</table>

B

NG  NG*  HG  HG*

pAkt  Akt

NG – Glucose 5 mmol/L
HG – Glucose 30 mmol/L
+ - Insulin 100 nmol/L
Supplemental Figures Legends

Supplemental Figure 1. *Schematic of Animal Study Design*

Supplemental Figure 2. *Insulin resistance model in vivo and in vitro*

(A) Representative western blots from the iliac artery of diabetic and non-diabetic rabbits two weeks after Alloxan or no injection, respectively, demonstrating reduction in Akt phosphorylation with preservation of Erk signaling.  (B) Representative western blots from human aortic endothelial cells (HAECs) after 72 hours exposure to hyperglycemia (30 mM) with and without 1 hour insulin (100 nM) compared to normoglycemia with and without insulin exposure. Note the reduction in Akt phosphorylation after stimulation with insulin after exposure to hyperglycemia versus normoglycemia.
Supplemental References


