Vascular Neointimal Formation and Signaling Pathway Activation in Response to Stent Injury in Insulin-Resistant and Diabetic Animals

Michael Jonas, Elazer R. Edelman, Adam Groothuis, Aaron B. Baker, Philip Seifert, Campbell Rogers

Abstract—Diabetes and insulin resistance are associated with increased disease risk and poor outcomes from cardiovascular interventions. Even drug-eluting stents exhibit reduced efficacy in patients with diabetes. We now report the first study of vascular response to stent injury in insulin-resistant and diabetic animal models. Endovascular stents were expanded in the aortae of obese insulin-resistant and type 2 diabetic Zucker rats, in streptozotocin-induced type 1 diabetic Sprague-Dawley rats, and in matched controls. Insulin-resistant rats developed thicker neointima (0.46±0.08 versus 0.37±0.06 mm²; P=0.05), with decreased lumen area (2.95±0.26 versus 3.29±0.15 mm²; P=0.03) 14 days after stenting compared with controls, but without increased vascular inflammation (ED1+ tissue macrophages). Insulin-resistant and diabetic rat vessels did exhibit markedly altered signaling pathway activation 1 and 2 weeks after stenting, with up to a 98% increase in p-ERK (anti-phospho ERK) and a 54% reduction in p-Akt (anti-phospho Akt) stained cells. Western blotting confirmed a profound effect of insulin resistance and diabetes on Akt and ERK signaling in stented segments, p-ERK/p-Akt ratio in stented segments uniquely correlated with neointimal response ($R^2=0.888$, $P=0.04$) in insulin-resistant and type 1 and 2 diabetic rats, but not in lean controls. Transfemoral aortic stenting in rats provides insight into vascular responses in insulin resistance and diabetes. Shifts in ERK and Akt signaling related to insulin resistance may reflect altered tissue repair in diabetes accompanied by a shift in metabolic/proliferative balance. These findings may help explain the increased vascular morbidity in diabetes and suggest specific therapies for patients with insulin resistance and diabetes. (Circ Res. 2005;97:725-733.)

Key Words: diabetes mellitus ■ insulin resistance ■ Akt ERK signaling ■ stent ■ restenosis
second model of diabetes, characterized by hyperglycemia and hypoinsulinemia (resembling type 1 diabetes), Sprague-Dawley rats, aged 13 weeks, were rendered diabetic by intravenously injection of streptozotocin (STZ, 50 mg/kg in citrate buffer, pH 4.5).12,13 Animals were considered diabetic after maintaining a plasma glucose level >300 mg/dL for a 2-week period, at which time aortic stenting was performed. These type 1 diabetic rats were compared with control Sprague-Dawley rats. All animals were from Charles River Laboratories (Wilmington, Mass). Animal care and procedures were in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care and the National Institutes of Health.

Abdominal Aortic Stenting and Tissue Harvest

Under anesthesia with inhaled isoflurane, a small incision was made in the right femoral artery, which was ligated. An arteriotomy was performed proximal to the ligature and a 0.014-in angioplasty guide wire was passed into the aorta. A 9-mm long endovascular steel stent (Nirflex, Medinol Inc) mounted on a 15-mm long x 2.5-mm diameter angioplasty balloon (Crossail, Guidant Inc) was passed via the arteriotomy into the abdominal aorta and deployed with a 1.5-second 8 atm inflation. The balloon and wire were removed and the femoral artery ligated. Heparin (100 U/kg intravenous) was administered before stenting and aspirin was added to drinking water beginning after stenting. Baseline signaling characteristics of nonstented, control aortae from Zucker fatty, diabetic, and lean rats (n = 3 from each group) were also studied.

Effect of Insulin Resistance and Diabetes on Metabolic Parameters and Neointimal Response

Zucker fatty rats were obese, with mild hyperglycemia and severe insulin resistance as evidenced by very high insulin levels. Zucker diabetic rats were more severely hyperglycemic, with significant hypoinsulinemia (Table). Insulin-resistant Zucker fatty, overtly hyperglycemic Zucker diabetic, and STZ-induced Sprague-Dawley diabetic rats, and these animals lost body mass (Table). Of the 35 Zucker rats, 18 were analyzed for protein expression by Western blotting (7 and 14 days) and 17 were analyzed morphometrically and immunohistologically (14 days). Similar morphometric and immunohistological analyses were performed on the 7 abdominal aortic stented segments of the Sprague-Dawley rats 14 days after stenting. Baseline signaling characteristics of nonstented, control aortae from Zucker fatty, diabetic, and lean rats (n = 3 from each group) were also studied.

Results

Thirty-five Zucker rats survived after stenting until planned euthanasia (12 Fatty, 10 Diabetic, and 13 Lean). Three died after successful aortic stenting: 2 from femoral vascular complications and 1 from internal hemorrhage after inadvertent stent deployment in the renal artery. Sprague-Dawley STZ-induced diabetic rats underwent successful aortic stenting after 2 weeks of maintained diabetic state (n = 4) and were compared with control Sprague-Dawley rats (n = 3). No stent thrombosis occurred during the study. Good wound healing was evident and all rats were bright and alert with normal eating and drinking patterns. Polyuria and polydipsia were evident in both Zucker diabetic rats and STZ-induced Sprague-Dawley diabetic rats, and these animals lost body mass (Table). Of the 35 Zucker rats, 18 were analyzed for protein expression by Western blotting (7 and 14 days) and 17 were analyzed morphometrically and immunohistologically (14 days). Similar morphometric and immunohistological analyses were performed on the 7 abdominal aortic stented segments of the Sprague-Dawley rats 14 days after stenting. Baseline signaling characteristics of nonstented, control aortae from Zucker fatty, diabetic, and lean rats (n = 3 from each group) were also studied.

<table>
<thead>
<tr>
<th>Metabolic Parameters</th>
<th>Zucker Fatty</th>
<th>Zucker Diabetic</th>
<th>Zucker Lean</th>
<th>Sprague-Dawley Diabetic</th>
<th>Sprague-Dawley Lean</th>
</tr>
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<tr>
<td>Weight, g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>578±46†</td>
<td>387±35</td>
<td>354±33</td>
<td>339±6</td>
<td>346±6</td>
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<tr>
<td>∆ at end point</td>
<td>80±23†</td>
<td>−27±27†</td>
<td>41±25</td>
<td>−31±23§</td>
<td>120±18</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>209±79</td>
<td>490±48†</td>
<td>195±40</td>
<td>495±120§</td>
<td>148±12</td>
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<tr>
<td>Hemoglobin A1C, %</td>
<td>4.28±0.61†</td>
<td>8.03±0.78†</td>
<td>3.38±0.63</td>
<td>7.2±0.69</td>
<td>4.4±1.9</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>15.37±3.16†</td>
<td>2.73±1.07</td>
<td>2.01±0.71</td>
<td>0.65±0.70e</td>
<td>3.50±1.01</td>
</tr>
<tr>
<td>Lipids, mg/dL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total cholesterol</td>
<td>196±37†</td>
<td>360±18*</td>
<td>80±4</td>
<td>181±20†</td>
<td>76±6</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>806±344†</td>
<td>649±102†</td>
<td>200±4</td>
<td>1496±30§</td>
<td>186±100</td>
</tr>
</tbody>
</table>

* P<0.05, †P<0.01 vs Zucker lean group; ‡P<0.05, §P<0.01 vs Sprague-Dawley lean group.
rats (8846 ± 106 cells/mm²) compared with the Lean animals (9764 ± 746 cells/mm²).

As in other models of stent-induced injury, macrophages (ED1) were identified within the neointima, typically clustered around stent struts. Fourteen days after stenting, slightly more macrophages were present in vessels from diabetic than lean animals (4.52 ± 0.39% versus 3.00 ± 0.73% of total cells, P < 0.02). In contrast, there was no difference between insulin-resistant fatty (3.02 ± 1.45% of total cells) and control lean rats (3.00 ± 0.73% of total cells, P = 0.43). Proliferating cells at 14 days (Ki-67, Figure 3) were 3-fold increased in the Zucker fatty compared with lean rats. Diabetic Sprague-Dawley rats exhibited less neointima than control animals 14 days after stenting (0.16 ± 0.06 versus 0.40 ± 0.05 mm², P < 0.01; Figure 2).

**Activation of ERK and Akt in Vessel Wall**

**Immunohistochemistry**

Cells with positive cytoplasmatic staining of p-Akt (anti-phospho Akt, Ser473) and positive cytoplasmatic and nuclear staining of p-ERK (anti-phospho ERK, Thr202/Tyr204) were identified in sequential sections (Figure 4). After 14 days, cells staining for p-Akt were reduced by 54%, and for p-ERK increased by 51% in Zucker fatty compared with Zucker lean rats. Stented segments of Zucker diabetic rats had a similar percent of p-Akt positive cells as compared with Zucker lean rats, whereas the fraction of p-ERK stained cells nearly doubled (Figure 5). The ratio of p-ERK to p-Akt stained cells was significantly higher in both Zucker fatty (3.5 fold, P < 0.01) and Zucker diabetic rats (2.2 fold, P < 0.05) as compared with Zucker lean rats. The p-ERK/p-Akt ratio within each artery was closely coupled with neointimal thickening in Zucker fatty and Zucker diabetic rats (Figure 6) but not Zucker lean controls.

In the Type 1 diabetic Sprague-Dawley rats, as in the Zucker fatty and Zucker diabetic rats, the activation of ERK relative to Akt in stented aortae was predictive of neointimal response. This relationship was not seen in either control group (Sprague-Dawley normal or Zucker lean rats; Figure 6).

**Western Blot Analysis**

The effect of stent implantation on ERK and Akt activation was further examined by Western blotting of protein extracts isolated from stented aortic segments after 7 and 14 days. The total amount of ERK and Akt was similar among the 3 groups of Zucker rats. Analysis of control aortae from nonstented animals showed similar baseline phosphorylation levels of Akt at Ser473 and ERK at Thr202/Tyr204 for fatty, diabetic, and lean Zucker rats (supplemental Figure I). In contrast, in animals subjected to vascular stenting, phospho-activation of ERK and Akt at the stented aortic segment was significantly altered by insulin-resistant or diabetic states (Figure 7). Phospho ERK levels were increased and phospho Akt levels reduced in stented vessels of Zucker fatty and diabetic rats in comparison with Zucker lean control rats after 7 and 14 days. Compared with Zucker lean control rats, the p-ERK/p-Akt ratio in stented vessels from Zucker fatty rats was increased 2.5 fold after both 7 and 14 days (P < 0.05), and in Zucker diabetic rats 3.5 fold at 7 days and 2.2 fold at 14 days (P < 0.01, Figure 7).

**Discussion**

Controlled vascular injury using minimally invasive implantation of endovascular stents enabled analysis of areas of...
injury in diabetic animals. The vascular responses to stenting were quantitatively and mechanistically different in obese, insulin-resistant Zucker fatty rats and Zucker diabetic rats compared with lean, normal controls. We postulated a priori that aberrations in insulin signaling would be accompanied by an imbalance in relative metabolic to proliferative signaling, and specifically examined the proportional activation of the ERK and Akt signaling pathways. The ratio of the phosphorylated forms of these 2 signaling elements predicted with high precision the increased neointimal response to stent implantation observed in insulin-resistant and diabetic animals.

Insulin Resistance
Insulin resistance, defined by compensatory hyperinsulinemia required to maintain normoglycemia, arises from defective glucose uptake by adipose tissue and skeletal muscle.18 Two major signaling pathways mediate the primary actions of insulin.19,20 The pathway that involves insulin receptor substrate proteins, phosphatidylinositol 3-kinase (PI3K), and Akt regulates the metabolic actions of insulin. The second signaling pathway, responsible for the mitogenic aspects of insulin action, proceeds through ras, raf and leads to activation of the mitogen-activated protein kinase (MAPK) isoform ERK. Whereas the metabolic Akt pathway is impaired in insulin resistant states, the ERK pathway remains intact, possibly serving to explain the preserved growth and mitogenic effects of insulin in insulin resistance. This signaling pathway’s selective resistance to insulin, with defective Akt activation and preserved ERK signaling, has been demonstrated in vascular smooth muscle cells (VSMCs), endothelial cells, and human skeletal muscle.21–23 Similarly, selective resistance to insulin signaling by the PI3K-Akt but not the MAPK-ERK pathway has been observed in vascular tissues of fatty Zucker rats and in aortae of spontaneously hypertensive rats.24,25 The signaling pathways active in the vascular response to stent injury of insulin-resistant animals have not been previously studied.

Using immunohistochemical methods and Western blotting, we found that normal, insulin-resistant, and overtly type 1 and 2 diabetic animals have significantly different relative activation of the ERK and Akt signaling pathways after controlled vascular injury. In the vessel wall of stented insulin-resistant and overtly diabetic rats, the ratio of cells exhibiting the activated phospho-forms of ERK versus Akt was increased compared with normal, lean controls. Western blotting confirmed that total vessel wall levels of p-Akt were reduced and of p-ERK increased in insulin resistant and diabetic rats seven and 14 days after stenting.

We also found that the p-ERK/p-Akt ratio correlated directly with the degree of stent-induced neointimal thickening (Figure 6) in insulin-resistant and diabetic animals. This correlation stands in contrast to the absence of any exaggerated inflammatory cell recruitment in Zucker fatty animals.
Inflammatory cell recruitment has been causally linked to stent-induced neointimal thickening in nondiabetic animals.26–28 Though neointimal thickening was increased in the insulin-resistant obese Zucker fatty rats, these animals did not have an increase in invading macrophages after 14 days. Taken together, these data support differential signaling activation, with a pathway-selective response to stent implantation in vessel walls of insulin resistant and diabetic animals underlying their neointimal responses.

ERK and Akt Pathways in Vascular Injury

The ras-raf-MAPK-ERK pathway is a key transducer of mitogenic signals from the plasma membrane to the nucleus in many cell types. Balloon denuding arterial injury increases medial VSMC proliferation, with associated upregulation of MAPK phosphorylation and activation, in both euglycemic animals and in hyperglycemic animals with chemically-induced type 1 diabetes.29,30 Inhibition of ras or MAPK genes prevents neointimal formation after balloon angioplasty.31 Furthermore, ERK downstream to ras seems to be a major intracellular target of insulin’s action on cultured VSMCs exposed to high doses of insulin. In vivo transfection of a ras-negative mutant gene reduced neointimal formation after balloon injury in nondiabetic rats rendered hyperinsulinemic by pancreatic islet cell implantation.12 In nondiabetic animals, activation of both the ERK and Akt signaling pathways was evident early after balloon injury, but returned to baseline after 7 days.32,33 Inhibition of ERK activity with PD98059 and of Akt with the PI3K inhibitor Wortmannin reduced early medial SMC replication but had no affect on intimal SMCs when given at a later time point.32,33 Reduced SMC proliferation with a resultant decrease in neointimal response was also achieved by Akt inhibition using local transfection of a dominant-negative Akt mutant at the time of rat carotid balloon injury.34 In a model of open-field surgical insertion of vascular stents into nondiabetic rats, aortic Akt activation was
evident after stenting, and its inhibition by systemically administered agents reduced neointimal response.35

**ERK Versus Akt in Stented Aortas**

We found phosphorylation of Akt and ERK extended to 14 days after aortic stenting. This prolonged activation, not seen with balloon denudation,32,33 may arise from the deeper and more sustained vascular injury induced by stenting. It is notable that in nondiabetic animals, inhibition of either the MAPK-ERK or the PI3K-Akt pathways resulted in reduced neointimal response.12,31,34,35 Although both signaling pathways can regulate cell proliferation and growth, we found that the balance between ERK and Akt activation, as indicated by the p-ERK/p-Akt ratio, correlated strongly with the vascular neointimal response in insulin-resistant and type 1 and 2 diabetic animals, but not in control rats. In contrast, activation patterns of each individual pathway (increase in ERK or decrease in Akt activation) failed to predict the degree of neointimal formation. We speculate that an increase in mitogenic signaling through ERK activation leading to increased cell proliferation and deprivation of the vasculature of the protective effect afforded by Akt activation interact to govern the neointimal response in insulin-resistant and diabetic animals:

Because both signaling pathways are downstream to insulin binding, specific resistance to the Akt pathway in the insulin resistant state, coupled with characteristically high circulating insulin levels, may cause shunting toward the ERK pathway. This would in turn lead to increased cell proliferation and migration. This mechanism may not only increase mitogenic signaling through ERK activation but also may deprive the vasculature of the protective effects afforded by Akt activation. Akt may well play a role in vascular-protective effects, as endothelial production of nitric oxide is induced by Akt activation and reduced by Akt inhibition.36,37 Furthermore, Akt inhibition coupled with excess insulin concentration may lead to increased expression of cellular adhesion molecules, with increased monocyte rolling and arrest.38 In VSMCs, Akt activation is probably necessary for
maintaining a quiescent, fully differentiated phenotype rather than a migratory, proliferative one. In the setting of insulin resistance, stent implantation may further disrupt the balance that normally regulates vasodilatation, vasculature inflammatory responses, and VSMC proliferation/migration by differentially shunting signaling from Akt to ERK activation.

Mammalian target of Rapamycin (mTOR) activation may be among the factors critical to regulation of cell growth and vascular repair, particularly in relation to insulin signaling. Although binding of insulin to its receptor may lead to phosphorylation and activation of mTOR via PI3K and Akt phosphorylation, a parallel, nutrient-sensitive pathway may primarily govern cell growth through mTOR. It has been shown that mTOR exists in various distinct complexes within cells, while characteristics of this pathway’s function and regulation continue to be revealed.

mTOR regulation and activation is an example of a key pathway governing cell growth and proliferation in a complex manner. As important as any single regulatory pathway or protein may be, however, the end effect of cell growth and proliferation is increasingly felt to result from balanced activation of many parallel pathways. Our findings point to the ratio of activation of the ERK and Akt pathways, both downstream to the insulin receptor, as best reflecting the sum-total of the neointimal response in insulin-resistant and diabetic states.

Molnar et al. recently described a mouse model of diet-induced obesity/diabetes with marked endothelial dysfunction but without the expected increased neointimal formation in response to injury. These fat-fed diabetic mice had preserved insulin stimulated Akt (and endothelial nitric oxide synthase) phosphorylation, in contrast to the Zucker insulin resistant rats. We show that this defective Akt pathway, as part of the imbalance in the ERK/Akt signaling, predicted with high precision the increased neointimal response to stent implantation in insulin-resistant animals. It may be postulated that a preserved phospho-ERK/Akt ratio in the vessels of the obese mice model described by Molnar et al, explains their lack of increased neointimal response to vascular injury.

Neointimal Response in the Severely Hyperglycemic State

Interestingly, the overtly diabetic Zucker rats exhibited a similar degree of neointimal formation to lean rats, (both significantly less than in insulin-resistant Zucker fatty rats). Furthermore, the neointimal response of the hyperglycemic, hypoinsulinemic, type 1 diabetic Sprague-Dawley rats was significantly reduced compared with lean controls. Blunted neointimal formation seen in rats with uncontrolled hyperglycemia could be secondary to a combination of factors, including a general catabolic state, as reflected by weight loss during the study period (Table), or hypoinsulinemia itself—absolute, as in type 1 diabetes, or relative, as in overt type 2 diabetes.

Uncontrolled, severe hyperglycemia interferes with the maintenance of a positive energy balance, often resulting in weight loss. Previous studies of balloon injury in uncontrolled, severely hyperglycemic animals have repeatedly shown neointimal responses to be equal or even reduced when compared with controls. The weight loss noted in both the Zucker diabetic and the STZ-induced Sprague-Dawley diabetic rats in the present study probably represent a general catabolic state, contributing to blunted neointimal formation.

Notwithstanding this, the basic mechanism governing the vascular response of the overtly diabetic Zucker rats was similar to the fatty, insulin-resistant animals, namely increased ERK pathway activation with blunted Akt signaling. The p-ERK/p-Akt ratio was similarly predictive of neointima formation in the insulin-resistant Zucker rats, with or without overt hyperglycemia, but not in the lean controls. Furthermore, significantly decreased neointimal formation seen in type 1 diabetic Sprague-Dawley rats also showed the same correlation between ERK versus Akt activation and neointimal response. These findings may highlight the role of hyperinsulinemia and insulin resistance as opposed to hyperglycemia per se as drivers of increased neointimal response.

Sprague-Dawley STZ-treated rats were rendered severely hypoinsulinemic, similar to the type 1 form of diabetes. Zucker diabetic rats have a form of advanced type 2 diabetes, with pancreatic beta cell failure with relative hypoinsulinemia manifested as hyperglycemia due to significant tissue insulin resistance. As ERK and Akt signaling are both downstream to insulin binding, the hyperglycemia seen in overtly diabetic animals without accompanying hyperinsulinemia may not be sufficient to elevate the ratio of ERK to Akt activation so as to result in increased neointima formation. Thus, the roles of hyperglycemia and hyperinsulinemia in neointimal formation have not yet been fully resolved. Future work may elucidate how treating overtly hyperglycemic Zucker diabetic rats with various agents might affect vascular responses to mechanical injury.

Limitations

Though our data consistently demonstrate low variance and our hypothesis is supported by multiple techniques, the mechanisms active in rats’ vascular responses to stenting may not be fully predictive of those observed in humans. In addition, the longstanding uncontrolled hyperglycemia seen in the overtly diabetic rats is not representative of the human type 2 diabetic state. Nevertheless, the Zucker rat model is well studied and shares some salient features with the metabolic syndrome of human disease (obesity, hyperlipidemia, elevated insulin levels). The differential affect of insulin resistance on the Akt- and ERK-mediated signaling is shared by the Zucker rats and human subjects, at least in muscle tissue. Extension of our findings, possibly manipulating ERK and Akt pathways with systemic or local agents, will provide important insights into the pathophysiology of the vascular response to injury in insulin resistance and diabetes. An important area of future study will be the full delineation of inflammatory responses active in insulin-resistant and diabetic states.

Conclusions

Transfemoral aortic stenting in rats allows insight into vascular responses to injury in obese, insulin resistant, and diabetic states. Shifts in ERK and Akt signaling related to
impaired intracellular pathway activation in the vessel wall may underlie the exaggerated tissue responses to stenting associated with diabetes. Such findings may aid development and evaluation of specific systemic or stent-based therapies for patients with insulin resistance and diabetes.

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References


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Histological and Immunohistochemical Analysis

Tissue and cell structures were identified in histological sections with Verhoeff’s tissue elastin and H&E stains. Neointimal and medial cross-sectional areas were measured by computer-assisted digital planimetry. For stented arteries, values from proximal, mid, and distal sections were averaged. Species-specific antibodies identified rat macrophages (1:100, ED1, MIB 5 clone, DAKO) and proliferating cells (1:50 Anti-rat Ki67, MIB 5 clone, DAKO). Analysis of phospho-Akt was performed with rabbit polyclonal p-Akt (1:25, Ser473, IHC specific) and phospho-ERK with rabbit polyclonal p-p44/p42 MAP kinase (1:25, Thr202/Tyr204) antibodies (Cell Signaling Technology, Beverly, MA). Labeled strepavidin-biotin immunoperoxidase methods were used in conjunction with heat-induced epitope retrieval. Sections were heated to 80°C in Target Retrieval Solution (DAKO Co.), blocked in 10% Fetal calf serum and then 5% BSA in PBS and incubated with the primary antibody. Biotinylated secondary antibody with horseradish peroxidase-labeled strepavidin from the DAKO LSAB 2 kit were used, together with diaminobenzidine and hydrogen peroxide chromogen substrate (DAKO). Slides were counterstained with hematoxylin and mounted. Negative controls were incubated with nonimmune rabbit or mouse IgG in place of primary antibody. Rat lymph node was used as a positive control. Overall cell density was calculated by dividing the number of nuclei by the intimal or medial area. Total immuno-positive cells (ERK, Akt, ED1 or Ki67) were counted and % positive cells were determined after dividing by number of total intimal or medial nuclei. The total (intima+media) % positive cells in the Fatty and diabetic animals were compared to the % positive cells in the lean control. For
each arterial section with staining for p-ERK and p-Akt as well as measurable neointima
the ratio of % positive p-ERK to % positive p-Akt was determined.

**Aortic Protein Extraction and Western Blotting Analysis**

A separate set of stented abdominal aortae were harvested for protein analysis by
Western blotting. After perfusion with NaCl, the adherent fat and connective tissues were
rapidly dissected away, the stent carefully removed, and the tissue snap-frozen with
liquid N₂. Tissue from each animal was later incubated in ice cold lysis buffer (20mM
Tris, 150mM NaCl, 1% Triton-X-100, 1% Deoxycholate, 0.1% SDS, 2mM EDTA, 2mM
Na₃VO₄, 2mM PMSF, 50mM NaF, protease inhibitor pellet (complete, Roche) and 10%
Glycerol) and homogenized on ice with a homogenizer (Polytron, Brinkmann
Instruments, Inc.). Insoluble material was removed by centrifugation at 15000 g for 15
minutes at 4°C and the protein concentration measured by bicinchoninic acid assay
(Pierce). Samples were boiled for 5 minutes with Laemmlie sample buffer and equal
amounts of protein run on a 10% SDS-polyacrylamide gel, transferred to PVDF
membrane and blocked for 1 hour with 5% nonfat dry milk. The blots were incubated in
4°C overnight with anti Akt, anti p44/p42 MAP kinase (ERK) anti-phospho-Akt
(Ser473) and anti phospho-p44/p42 MAP kinase (ERK) (Thr202/Tyr204) (1:1000, Cell
Signaling). Horseradish peroxidase-labeled IgG secondary antibodies (1:2000, Santa
Cruz) with an enhanced chemiluminescence kit (PerkinElmer) were used to visualize the
protein bands. For quantification, images were analyzed by a densitometer. For each
arterial segment the ratio of p-ERK/p-Akt was calculated.
Blood Chemistry Assay

Blood samples were taken from Zucker rats prior to stenting and at sacrifice. Blood glucose measurements in the Sprague Dawley rats were further determined at baseline, post induction and weekly thereafter. Blood glucose was measured with a standard portable glucometer. Serum insulin was determined by a rat insulin ELISA (Linco). Serum lipids were measured in a routine diagnostic analyzer using enzymatic colorimetric assays, hemoglobin A1C levels were measured by Diatrac electrophoresis.

Statistical analysis

All data are presented as mean±SD. Statistical comparisons were analyzed by a Student’s t-test for two group comparisons. Values of p<0.05 were considered significant. Association of p-ERK/p-Akt ratio to neointimal response was tested with linear and non linear regressions, and the best fit was retained.
**Online Figure 1:** Western immunoblots with anti p-Akt(Ser473), anti p-ERK (Thr202/Tyr204), anti Total Akt and anti Total ERK of extracts from non-stented aortic segments of Zucker Lean control, Zucker Diabetic, and Zucker Fatty rats. Bar graph shows densitometric analysis of western blot data: Data are expressed as relative to Lean control.
Online Figure 1

The image shows a bar graph comparing the expression levels of p-ERK and p-Akt in fatty, diabetic, and lean groups. The graph indicates that p-ERK is significantly higher in the fatty group compared to the diabetic and lean groups. Similarly, p-Akt shows a trend with the fatty group having a higher expression level than the diabetic and lean groups.