Diabetes Induces Endothelial Dysfunction but Does Not Increase Neointimal Formation in High-Fat Diet Fed C57BL/6J Mice

Judit Molnar, Shuiqing Yu, Nino Mzhavia, Clara Pau, Igor Chereshnev, Hayes M. Dansky

Abstract—Studies of diabetic vascular disease have traditionally used murine models of type 1 diabetes and genetic models of type 2 diabetes. Because the majority of patients with type 2 diabetes have diet induced obesity, we sought to study the effect of diabetes on arterial disease in a mouse model of diet induced obesity/diabetes. C57BI/6 mice fed a high-fat diet for 9 weeks developed type 2 diabetes characterized by elevated body weight, hyperglycemia, and hyperinsulinemia. Arteries from diabetic mice exhibited a marked decrease in endothelium-dependent vasodilation, a modest decrease in endothelium independent vasodilation, and an increase in sensitivity to adrenergic vasoconstricting agents. Insulin stimulated protein kinase B (akt) and endothelial nitric oxide synthase (eNOS) phosphorylation were preserved in arteries from diabetic mice; however, eNOS protein dimers were markedly diminished. Arterial nitrotyrosine staining indicated that increased levels of peroxynitrite contributed to eNOS dimer disruption in the diabetic mice. The abnormal vasomotion was not an acute response to the high-fat diet, as short term high-fat diet feeding had no effect on endothelium dependent dilation. A trend toward smaller neointimal lesions was noted in high-fat diet fed mice after femoral artery wire denudation injury. In summary, disrupted eNOS dimer formation rather than impaired insulin mediated eNOS phosphorylation contributed to the endothelial dysfunction in diet induced obese/diabetic mice. The lack of an increase in neointimal formation indicates that additional diabetes associated parameters (such as hyperlipidemia and atherosclerotic vascular disease) may need to be present to increase neointimal formation in this model. (Circ Res. 2005;96:1178-1184.)

Key Words: diabetes ■ mice ■ endothelial nitric oxide synthase ■ arterial injury ■ high-fat diet ■ endothelial dysfunction.

Type 2 diabetes is a risk factor for endothelial dysfunction, atherothrombotic disease, and complications following acute ischemic events and revascularization procedures. Diabetes associated vascular disease affects multiple vascular beds, displays a significant inflammatory component, and is associated with increased neointimal formation after percutaneous coronary interventions. Although drug eluting stents have markedly decreased the overall incidence of restenosis, patients with diabetes still have a higher incidence of restenosis after receiving rapamycin coated stents. The fact that a large clinical trial failed to show a significant reduction in cardiovascular disease end points with tight control of blood glucose indicates that the interactions between diabetes and the arterial wall are complex and require additional experimental and clinical study.

The cellular and molecular mechanisms underlying diabetic vascular disease remain poorly understood. Hyperglycemia, insulin resistance, dyslipidemia, hypertension, and advanced glycation endproducts have all been implicated in the pathogenesis of accelerated arterial disease in patients with diabetes. The fact that experimentally induced hyperglycemia and hyperinsulinemia can reduce endothelium dependent vasodilation suggests that these metabolic parameters can have direct effects on the arterial wall. Reduced endothelium derived nitric oxide (NO) is thought to contribute to endothelial dysfunction, atherosclerosis, and restenosis through the loss of the vasodilatory, anti-inflammatory, and antiproliferative properties of nitric oxide, respectively. Reduced endothelial production of NO depends on a delicate balance between nitric oxide production via endothelial nitric oxide synthase (eNOS) and inactivation by reactive oxygen species such as superoxide. Endothelial production of nitric oxide requires the formation of eNOS homodimers and the phosphorylation of specific eNOS residues by protein kinase B (akt).

Many of the existing mouse and rat models have used streptozotocin or other methods to induce a type 1 diabetes syndrome to study vascular phenotypes such as endothelial...
dysfunction, atherosclerosis and restenosis. Streptozotocin treatment often increases plasma lipids in atherosclerosis-prone, hyperlipidemic mice. This can make it difficult to determine whether increases in atherosclerosis are because of diabetes or worsening of the lipoprotein profile. Because the majority of patients with type 2 diabetes are either overweight or obese, we sought to create a mouse model of diet induced obesity and diabetes with cardiovascular phenotypes. We determined whether hyperglycemia and hyperinsulinemia in the absence of marked elevations in plasma lipids would be sufficient to impair endothelial function and increase neointimal formation in response to arterial injury. Disruption of eNOS protein dimers without significant alterations in insulin mediated ENOS phosphorylation contributed to the reduced vasorelaxation in high-fat fed diabetic mice. However, neointimal size in response to arterial injury was decreased in high-fat fed mice. These data suggest that the metabolic defects in this mouse model are sufficient to impair endothelial function but other factors such as hyperlipidemia and atherosclerotic disease may need to be present to increase neointimal formation in response to arterial injury.

Materials and Methods

C57BL/6 male mice, 4 to 6 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, Me). Mice were maintained in a temperature-controlled barrier facility with a 12-hour light/dark cycle and were given free access to food and water. Mice were fed rodent chow until the age of 8 to 10 weeks. At that time, mice were either maintained on the chow diet (Purina Picolab 5053) containing 45% fat and 72% carbohydrate or started on a high-fat diet (BioServ Industries, Frenchtown, NJ S3282 or equivalent diet from Harlan Teklad TD03584, Indianapolis, Ind). The high-fat diets contained 35% fat (primarily lard) and 37% carbohydrate (primarily sucrose) without cholate supplementation. All procedures on mice were approved by the Institutional Animal Care and Use Committee at the Mount Sinai School of Medicine and were performed at the Mount Sinai School of Medicine. A detailed description of the experimental methods is available in the online data supplement at http://circres.ahajournals.org.

Results

Adult C57BL/6 male mice were fed a high-fat diet to induce obesity and diabetes. After 9 weeks of feeding, body weight and plasma leptin were elevated in high-fat compared with chow diet fed mice (see online Table I), and strong correlations between body weight and leptin were observed (Figure 1A). High-fat diet fed mice developed type II diabetes characterized by elevations in fasting plasma glucose and insulin (see online Table I) and by an impaired intraperitoneal glucose tolerance test (Figure 2A). Total cholesterol concentration was elevated, and plasma triglyceride concentration was reduced in high-fat diet fed mice (see online Table I). Differences in body weight were maintained after 19 weeks of feeding (42 ± 2 versus 29 ± 1 g, P = 0.0008; n = 8 to 9 per group) and were associated with significant elevations in fat pad mass (epididymal fat: 2.2 ± 0.24 versus 0.51 ± 0.08 g, P = 0.00001; perirenal fat: 1.3 ± 0.22 versus 0.15 ± 0.04 g, P = 0.00004, n = 4 per group). Strong correlations between body weight and metabolic parameters were noted in high-fat fed mice (Figure 1B through 1D). These data closely parallel human data demonstrating that adiposity correlates with the development of type II diabetes.

Arterial vasodilation to acetylcholine was markedly attenuated in high-fat diet fed mice (Figure 3A). Arteries from high-fat diet fed mice were markedly less sensitive (6.4 ± 0.13 versus 7.0 ± 0.07, P = 0.0005, n = 5 to 7 per group), and maximum relaxation response (28.4 ± 1.7 versus 60.6 ± 1.2%, P < 0.0001, n = 5 to 7 per group) was significantly lower compared with arteries from chow diet fed mice. There was a small, but significant decrease in relaxation to sodium nitroprusside in arteries from high-fat fed mice (Figure 3B), but comparisons of relaxation at any given dose of sodium nitroprusside in arteries from high-fat fed mice (Figure 3B), but comparisons of relaxation at any given dose of sodium nitroprusside in arteries from high-fat fed mice (Figure 3B), but comparisons of relaxation at any given dose of sodium nitroprusside in arteries from high-fat fed mice (Figure 3B), but comparisons of relaxation at any given dose of sodium nitroprusside in arteries from high-fat fed mice (Figure 3B), but comparisons of relaxation at any given dose of sodium nitroprusside in arteries from high-fat fed mice (Figure 3B), but comparisons of relaxation at any given dose of sodium nitroprusside in arteries from high-fat fed mice (Figure 3B), but comparisons of relaxation at any given dose of sodium nitroprusside in arteries from high-fat fed mice (Figure 3B), but comparisons of relaxation 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nitroprusside were not statistically significant on posttest analysis. Sensitivity to sodium nitroprusside in high-fat diet fed mice was unchanged (6.75 ± 1.47 versus 6.75 ± 1.86, p = ns, n = 5 to 7 per group), but the maximum relaxation response (46.7 ± 1.5 versus 67.0 ± 1.9%, P < 0.0001, n = 5 to 7 per group) was lower in arteries from high-fat diet fed mice. Arteries from high-fat diet fed mice had increased vasoconstriction to phenylephrine (Figure 3C) and norepinephrine (data not shown). Sensitivity to phenylephrine (6.1 ± 0.02 versus 5.7 ± 0.04, P < 0.0001, n = 4 to 5 per group) and maximal wall tension to phenylephrine (1.5 ± 0.01 versus 1.2 ± 0.02 mN/mm, P < 0.0001, n = 4 to 5 per group) were significantly increased in arteries of high-fat diet fed compared with chow diet fed mice.

Insulin mediated phosphorylation of akt and eNOS were measured in the arteries of high-fat diet fed diabetic mice to determine whether decreased arterial insulin signaling (insulin resistance) was responsible for impaired NO release and vasodilation. Intravenous insulin increased akt phosphorylation in the femoral artery of chow diet and high-fat diet mice (F = 42.15; P < 0.0001; Figure 4A and 4C). The high-fat diet had no significant effect on baseline nor insulin induced akt phosphorylation in the femoral artery (Figure 4A and 4C). In the aorta, there was a small but significant interaction between the effect of the diet on insulin induced akt phosphorylation in the aorta (F = 6.0, P = 0.03, Figure 4E and 4G). Although insulin induced akt phosphorylation in both diet groups, the insulin mediated induction in aortic akt phosphorylation was slightly greater in chow diet fed mice compared with high-fat diet fed mice (3.3 versus 2.8 fold) (Figure 4G).

Insulin increased eNOS phosphorylation in the femoral artery of both chow and high-fat diet fed mice (F = 29 for overall effect of insulin; P = 0.0002; Figure 4B and 4D). There was a trend toward an effect of the diet on eNOS phosphorylation (F = 3.6, P = 0.08). In chow diet fed mice, eNOS phosphorylation in the femoral artery increased 2.5 fold (P < 0.001; Figure 4B). The baseline eNOS phosphorylation was higher in high-fat compared with chow diet fed mice (P < 0.05), so that there was no significant increase in insulin mediated eNOS phosphorylation in high-fat fed mice (Figure 4B and 4D). Although there was a greater effect of insulin on eNOS phosphorylation in the femoral arteries of chow versus high-fat diet fed mice, the absolute level of insulin induced eNOS phosphorylation was unchanged. In contrast, eNOS phosphorylation in the aorta did not change with insulin injection in both diet groups (Figure 4F and 4H).

Previous studies have shown that the formation of eNOS homodimers is necessary for enzymatic activity and that peroxynitrite formation can lead to disruption of the eNOS zinc thiolate cluster. We evaluated the relative amount of eNOS dimer in the femoral artery and aorta of high-fat diet induced diabetic mice. In arteries of chow diet fed mice, eNOS dimer formation was detected; however, in the diabetic mice, eNOS dimers were nearly absent in aorta (Figure 5A) and femoral arteries (data not shown). Immunostaining for nitrotyrosine (an indicator of peroxynitrite induced tyrosine nitrosylation) was performed on aortic sections from chow and high-fat fed mice (n = 3 of each diet group). The intensity and extent of nitrotyrosine immunostaining was significantly enhanced in the aorta of the diabetic mice compared with nondiabetic control mice (Figure 5B and 5C). These results strongly suggest that endothelial dysfunction observed in the arteries of the diabetic mice was mediated by peroxynitrite disruption of eNOS dimers, leading to reduced NO production.

In order to distinguish the direct effect of diet from the effect of diabetes, mice were fed a chow or high-fat diet for 1 week. At this time point, there were no significant differences in body weight, glucose, and insulin (data not shown). There was no statistical difference in glucose tolerance although the area under the curve (AUC) was greater in high-fat diet fed mice (Figure 2B). Except for a very small decrease in endothelium independent vasodilation, there were no differences in the response to acetylcholine and norepinephrine in mice fed the high-fat diet for 1 week (Figure 6). These results demonstrate that short term feeding of the high-fat diet did not impair endothelium dependent vasodilation in this model.

Bilateral femoral artery endovascular wire injury was performed to determine whether diabetes would increase...
neointimal formation. There was a trend toward decreased intimal size in high-fat fed diabetic mice (Figure 7A, 7D and 7E). There was a small, but significant increase in medial size in high-fat diet fed diabetic mice (Figure 7B), so that the intimal/medial (I/M) ratio decreased in high-fat diet fed diabetic mice (7C). Thus, despite apparent metabolic derangement and impairment in endothelium dependent vasodilation, neointimal formation in response to arterial injury was not increased in high-fat diet fed diabetic mice.

Discussion
Feeding of a high-fat, sucrose containing diet to C57Bl/6 mice resulted in a type 2 diabetes phenotype characterized by an increase in body weight and adipose mass, hyperglycemia and hyperinsulinemia. Previous studies have noted insulin resistance in high-fat fed mice using clamps and isotopic methods. In contrast to the elevations in plasma triglycerides and free fatty acids frequently observed in patients with diabetic dyslipidemia, feeding of the high-fat diet to C57BL/6 mice resulted in small increases in plasma cholesterol, no changes in circulating free fatty acids, and a decrease in plasma triglycerides. The decrease in plasma triglycerides in mice fed high-fat diets without cholate has been observed in previous reports, but the mechanism has not been elucidated. Because elevations in plasma triglycerides in humans has been associated with consumption of high-carbohydrate diets, the lower carbohydrate content in the high-fat versus the chow diet could have been responsible for the decrease in plasma triglyceride levels in the high-fat diet fed mice. Hyperlipidemia has been previously shown to induce endothelial dysfunction and increase neointimal formation in mouse models. Therefore, in the current study, effect of hyperglycemia and hyperinsulinemia on the vascular phenotypes could be determined.

A reduction in endothelium dependent vasodilation has been observed in patients with obesity/insulin resistance and type 2 diabetes and a few studies have also noted reduced endothelium dependent vasodilation. In the current study, 2 potential mechanisms of reduced eNOS function were explored to explain the reduced endothelium dependent vasodilation in the model of high-fat diet induced obesity/diabetes. The first mechanism was that defective insulin signaling or insulin resistance in the arterial wall led to reductions in akt mediated eNOS phosphorylation. Akt appears to be the principal kinase that phosphorylates eNOS and results in endothelium dependent vasodilation. Adenovirus mediated delivery of a dominant negative akt resulted in abolishment of acetylcholine induced endothelium dependent relaxation of mouse arteries. Except for minor differences, insulin mediated akt and eNOS phosphorylation were preserved in the arteries of the diabetic mice in the current study. Both akt and eNOS phosphorylation have been examined in methods.
a small number of studies of arteries from murine diabetic models. Depending on the methodology used, either increased or decreased akt phosphorylation has been observed in the aorta of obese Zucker fatty rats.38,39 One study noted an increase in baseline eNOS 1179 phosphorylation in the aorta of the obese Zucker rat.38 Based on the data in the current study, it is unlikely that arterial insulin resistance contributes to the reductions in endothelium dependent vasodilation in the diet induced model of obesity/diabetes by reducing eNOS phosphorylation.

The second mechanism was that enhanced reactive oxygen stress in the arterial wall of diabetic mice led to the disruption of eNOS dimers and reduced NO production. Hyperglycemia increases superoxide generation in endothelium by several different pathways,40 and superoxide is known to inactivate nitric oxide and produce peroxynitrite.16 Peroxynitrite can disrupt eNOS protein dimers through oxidation and displacement of the zinc metal ion.19 Depletion of eNOS cofactors such as tetrahydrobiopterin can also lead to the uncoupling of eNOS and be an additional source of superoxide.41,42 The overproduction of asymmetric dimethyl arginine, a competitive inhibitor of l-arginine, can also reduce the production of nitric oxide.43 In the present study, eNOS protein dimers were nearly absent in the high-fat fed diabetic mice. Because eNOS dimer formation is necessary for eNOS activity, the decrease in eNOS dimers will lead to reductions in eNOS activity in the arterial wall of high-fat fed diabetic mice. Cohen and coworkers first demonstrated that peroxynitrite can disrupt eNOS dimers through disruption of the zinc thiolate cluster.19 They also demonstrated the lack of protein dimers in the hearts of streptozotocin treated LDLR knockout mice.19 In the present study, the increased nitrotyrosine staining in the arteries from the diabetic mice demonstrates that type 2 diabetes is associated with the production of arterial reactive nitrogen species such as peroxynitrite. Although we did not directly measure peroxynitrite in the arterial wall in this study, other studies have documented increased superoxide in the arteries of diabetic mice.44 Overall, these data suggest that enhanced oxidant stress in the arteries of mice with type 2 diabetes results in increased formation of peroxynitrite, disruption of eNOS dimers, and reduced endothelium dependent vasodilation.

Short term feeding of the high-fat diet (1 week) did not result in alterations in metabolic parameters, and arterial vasomotion was unchanged compared with chow diet fed mice. These data demonstrated that the high-fat diet itself had no early direct effects on arterial vasomotion and suggested an association between metabolic abnormalities and endothelial dysfunction. Initial studies in humans indicated that serum triglycerides increase transiently after a single high-fat meal and are associated with impaired flow mediated dilation of the brachial artery in healthy subjects.45,46 Other studies have not demonstrated reduced endothelium dependent vasodilation after a high-fat meal or with an infusion of triglyceride emulsion.57,48 Studies have also suggested that the apparent decreases in flow mediated dilation after a high-fat meal may be simply because of high-fat mediated increases in baseline arterial diameter.48,49

The induction of diabetes in high-fat fed mice resulted in abnormal vasomotion but did not increase neointimal formation in response to femoral artery wire injury. In fact, there...
was a trend toward decreased intimal size in the high-fat fed mice. This decrease in femoral artery induced neointimal formation was consistent with our previous report showing a decrease in neointimal size in C57BL/Ks db/db diabetic mice; however, the magnitude of the effect on neointimal formation was much greater in db/db mice (~90% decrease).50 The potential role of leptin in neointimal formation is supported by the observation that exogenous leptin increases neointimal formation in the FeCl2 model.51 In diet induced obesity, leptin resistance could also have played a role in the decrease neointimal formation in the current study. Another possibility is that diabetes associated metabolic parameters (such as hyperglycemia and/or insulin resistance) reduces neointimal formation in the mouse by an unknown mechanism. The marked reduction in neointimal formation in the more severe model of diabetes (db/db mouse) suggests that this theory may be plausible. In contrast, two studies have demonstrated an increase in neointimal formation in response to balloon injury of the carotid arteries of obese type II diabetic Zucker rats.52,53 Both the db/db mouse and Zucker fatty diabetic rat have mutations in the leptin receptor which result in obesity, insulin resistance, and diabetes. Hyperglycemia appears to be more severe in the db/db mouse than the Zucker diabetic rat; however, hyperglycemia does not appear to alter neointimal formation murine models of type 1 diabetes.21,50,52 Increased neointimal formation in the Zucker diabetic fatty rats may relate to the severe hypertriglyceridemia (~500 mg/dL) in the Zucker fatty diabetic rat whereas db/db mice that have normal plasma triglyceride concentrations.52 It is unclear whether the reduced endothelial dependent and/or independent vasomotion in the high-fat fed diabetic mice could have had an impact on the response to endovascular wire injury. Both physical training and estrogen have been shown to increase reendothelialization after arterial injury in rodent models in an eNOS dependent fashion54,55; however, these interactions have not been studied in the setting of diabetes. Further investigations will be needed to sort out the relationships among metabolic parameters and the response to arterial injury in the mouse and rat models.

In summary, high-fat diet feeding induces a type II diabetes phenotype with endothelial dysfunction. Disrupted eNOS dimer formation rather than impaired insulin mediated eNOS phosphorylation contributed to the endothelial dysfunction in diet induced obese/diabetic mice. The lack of increased neointimal formation suggests that other factors such as hyperlipidemia, atherosclerosis, and possibly factors relating to thrombosis may need to be present for diabetes to accelerate neointimal formation in the mouse. The lack of suitable mouse models of diabetic atherosclerosis and restenosis underscores the need for additional studies to work out basic pathways by which diabetes related metabolic factors affect the cells of the arterial wall.

Acknowledgments

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References


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Title: Diabetes induces endothelial dysfunction but does not increase neointimal formation in high fat diet fed C57BL/6J mice
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Online Supplement:

Materials and Methods:

Analytical procedures: Glucose levels were determined in fasted (4-6 hours) conscious mice from tail blood samples using a clinical glucometer (Glucometer Elite). Blood was collected from the retro-orbital venous plexus after isoflurane anesthesia for other biochemical measurements. Plasma was used for measurement of total cholesterol, triglyceride, and free fatty acids using enzymatic assays (Wako, Richmond, VA). Leptin and insulin were measured using ELISA assays from R&D (Minneapolis, MN) and from Alpco Diagnostics (Windham, NH).

For glucose tolerance tests, mice were fasted for 6 hours and were given an intraperitoneal injection of glucose (1mg/g body weight) in phosphate buffered saline (PBS). Blood glucose was determined from tail vein as described above at 0, 15, 30, 50, 90 and 120 min after injection.

Tissue collection: Mice were anesthetized with an intraperitoneal injection of ketamine and xylazine. The chest and peritoneal cavity were opened and the circulatory system was perfused via the left ventricle with phosphate buffered saline. Arteries were removed and processed for the various assays. For vascular studies, the left superficial femoral artery was removed and immediately placed in ice-cold physiologic salt solution (PSS). For arterial injury studies, arteries were isolated after paraformaldehyde fixation and processed as previously described.

Vascular function studies: All reagents were acquired from Sigma Chemical (St.Louis, MO). Femoral arteries with intact endothelium and similar dimensions were mounted on a small vessel wire myograph (Danish MyoTechnology, Aarhus, Denmark) as described previously.
were bathed in PSS at 37°C and aerated continuously with 5% CO2 / 95% O2 to achieve pH of 7.4. The arteries were set to a predetermined internal circumference, which has been shown to be the optimum caliber for studies of the peripheral vasculature. The startup protocol and evaluation of vessel viability was conducted as described previously. Concentration response curves were performed for potassium chloride, phenylephrine, norepinephrine, acetylcholine (endothelium dependent), sodium nitroprusside (endothelium-independent nitric oxide releasing agent). Due to differences in the arterial response to phenylephrine between the two diet groups, responses to vasodilator agents were determined following a stable contraction to KCl 50mM. Wall tension was expressed as milliNewton per millimeter (mN/mm) of artery length. Sensitivity to the agonist was expressed as the negative log of the effective concentration required to produce 50% of maximum effect (-log EC50). Sensitivity was calculated from each concentration response curve by fitting the Hill equation using Prism (GraphPad Software, San Diego, CA). The operator was unaware of treatment groups during data collection.

**Femoral artery wire injury:** Endovascular wire injury was performed in the femoral arteries of mice as previously described. Briefly, a 0.25mm angioplasty guidewire was used to denude and dilate the femoral arteries of mice. Mice were sacrificed 4 weeks after arterial injury. Intimal and medial areas were measured on sections of femoral artery stained with combined masson elastin stain using computer aided morphometry.

**Western Blotting:** Mice were fasted for 4 hours and injected with PBS or human recombinant insulin 3.8mU/g mouse body weight in 200ul PBS via the retroorbital plexus. Arteries were harvested 4 minutes after injection. Protein was resolved on 4-15%SDS-PAGE reducing gels (Bio-rad). Protein was transferred to PVDF membranes (Bio-Rad), blocked in 5% milk/tris buffered saline (TBS), and probed with primary antibodies overnight. Membranes were
washed, incubated with appropriate secondary antibodies conjugated to horseradish peroxidase, washed in TBS-tween 0.05%, incubated with ECL reagent (Amersham Biosciences, Piscataway, NJ) and exposed to x ray film (Denville Scientific, South Plainview, NJ). Blots were stripped and reprobed with additional primary antibodies. Quantitative densitometry was performed using chemiDoc XRS imager using Quantity one-4.5.0 software (Bio-rad). Anti-akt antibodies were obtained from Cell Signaling (Beverly, MA). Anti eNOS antibodies were obtained from BD Transduction Laboratories (Franklin Lakes, NJ). HRP conjugated secondary antibodies were obtained from Pierce (Rockford, IL). For detection of eNOS dimers, we sought to minimize artifacts due to ex vivo disruption of eNOS dimers by performing low temperature (four degrees celcius) polyacrylamide electrophoresis as previously described\(^7\). Pilot experiments demonstrated that incubation of the samples at 4, 25, and 37 degrees in the presence of SDS and 2.5% \(\beta\)-mercaptoethanol for up to 2 hours was not sufficient to denature the proteins in the arterial lysate, and eNOS protein did not migrate according to size on SDS-PAGE. When the protein lysates were heated to 65 degrees for 30 minutes in the presence of SDS and 2.5% \(\beta\)-mercaptoethanol, eNOS protein migrated according to its expected molecular weight. Although the heating procedure may have led to an underestimation of the absolute amount of eNOS dimers, all samples from both diet groups were processed simultaneously under the same conditions, and the relative differences between the diet groups persisted in all the experiments.

**Nitrotyrosine immunohistochemistry:** Sections from mice (n=3 from each diet group) were fixed with acetone and blocked with 8% goat serum in PBS for 1 hour. Sections were incubated with 1:100 dilution of rabbit anti-nitrotyrosine antibody (Upstate Technologies) in 4% goat serum overnight at 4 degrees. After washing, alexa 488 conjugated anti-rabbit antibody (Molecular Probes) was applied for 30 minutes, sections were washed and incubated with hoechst dye33258
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(Molecular Probes) and coverslipped. Fluorescence images were acquired using fixed exposure
times using a CCD camera attached to a Zeiss Axiovert inverted microscope. Negative and
positive control sections were preincubated with 3-nitrotyrosine (Sigma), or a peroxynitrite
solution (Upstate), respectively, to assure specificity of staining.

Statistics: Data are represented as mean ± standard error of the mean (sem). Significance was
assessed by two-tailed Student’s t-test for parametric data at p<0.05. Bonferroni post tests were
utilized after 2 way analysis of variance (ANOVA) to detect differences between groups.

Statistical tests were performed using Prism software (GraphPad).
Online Table I

<table>
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<tr>
<th>Diet</th>
<th>Period of feeding</th>
<th>Weight</th>
<th>Leptin</th>
<th>Glucose</th>
<th>Insulin</th>
<th>TC</th>
<th>TG</th>
<th>FFA</th>
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<tbody>
<tr>
<td>chow</td>
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<td>56±5(20)*</td>
<td>226±9(20)*</td>
<td>2.5±0.36(25)*</td>
<td>139±6(10)*</td>
<td>14±1(11)*</td>
<td>0.47±0.06(10)</td>
</tr>
</tbody>
</table>

Data expressed as mean ± standard error of the mean with the number of mice in parentheses. *p<0.0001


Title: Diabetes induces endothelial dysfunction but does not increase neointimal formation in high fat diet fed C57BL/6J mice

Judit Molnar¹, Shuiqing Yu², Nino Mzhavia², Clara Pau², Igor Chereshnev¹, Hayes M. Dansky ²

Online Supplement:

Materials and Methods:

Analytical procedures: Glucose levels were determined in fasted (4-6 hours) conscious mice from tail blood samples using a clinical glucometer (Glucometer Elite). Blood was collected from the retro-orbital venous plexus after isoflurane anesthesia for other biochemical measurements. Plasma was used for measurement of total cholesterol, triglyceride, and free fatty acids using enzymatic assays (Wako, Richmond, VA). Leptin and insulin were measured using ELISA assays from R&D (Minneapolis, MN) and from Alpco Diagnostics (Windham, NH).

For glucose tolerance tests, mice were fasted for 6 hours and were given an intraperitoneal injection of glucose (1mg/g body weight) in phosphate buffered saline (PBS). Blood glucose was determined from tail vein as described above at 0, 15, 30, 50, 90 and 120 min after injection.

Tissue collection: Mice were anesthetized with an intraperitoneal injection of ketamine and xylazine. The chest and peritoneal cavity were opened and the circulatory system was perfused via the left ventricle with phosphate buffered saline. Arteries were removed and processed for the various assays. For vascular studies, the left superficial femoral artery was removed and immediately placed in ice-cold physiologic salt solution (PSS). For arterial injury studies, arteries were isolated after paraformaldehyde fixation and processed as previously described ¹.

Vascular function studies: All reagents were acquired from Sigma Chemical (St.Louis, MO). Femoral arteries with intact endothelium and similar dimensions were mounted on a small vessel wire myograph (Danish MyoTechnology, Aarhus, Denmark) as described previously ². Vessels
were bathed in PSS at 37°C and aerated continuously with 5% CO₂ / 95% O₂ to achieve pH of 7.4. The arteries were set to a predetermined internal circumference, which has been shown to be the optimum caliber for studies of the peripheral vasculature. The startup protocol and evaluation of vessel viability was conducted as described previously. Concentration response curves were performed for potassium chloride, phenylephrine, norepinephrine, acetylcholine (endothelium dependent), sodium nitroprusside (endothelium-independent nitric oxide releasing agent). Due to differences in the arterial response to phenylephrine between the two diet groups, responses to vasodilator agents were determined following a stable contraction to KCl 50mM.

Wall tension was expressed as milliNewton per millimeter (mN/mm) of artery length. Sensitivity to the agonist was expressed as the negative log of the effective concentration required to produce 50% of maximum effect (-log EC50). Sensitivity was calculated from each concentration response curve by fitting the Hill equation using Prism (GraphPad Software, San Diego, CA). The operator was unaware of treatment groups during data collection.

**Femoral artery wire injury**: Endovascular wire injury was performed in the femoral arteries of mice as previously described. Briefly, a 0.25mm angioplasty guidewire was used to denude and dilate the femoral arteries of mice. Mice were sacrificed 4 weeks after arterial injury. Intimal and medial areas were measured on sections of femoral artery stained with combined masson elastin stain using computer aided morphometry.

**Western Blotting**: Mice were fasted for 4 hours and injected with PBS or human recombinant insulin 3.8mU/g mouse body weight in 200ul PBS via the retroorbital plexus. Arteries were harvested 4 minutes after injection. Protein was resolved on 4-15%SDS-PAGE reducing gels (Bio-rad). Protein was transferred to PVDF membranes (Bio-Rad), blocked in 5% milk/tris buffered saline (TBS), and probed with primary antibodies overnight. Membranes were
washed, incubated with appropriate secondary antibodies conjugated to horseradish peroxidase, washed in TBS-tween 0.05%, incubated with ECL reagent (Amersham Biosciences, Piscataway, NJ) and exposed to x-ray film (Denville Scientific, South Plainview, NJ). Blots were stripped and reprobed with additional primary antibodies. Quantitative densitometry was performed using chemiDoc XRS imager using Quantity one-4.5.0 software (Bio-rad). Anti-akt antibodies were obtained from Cell Signaling (Beverly, MA). Anti eNOS antibodies were obtained from BD Transduction Laboratories (Franklin Lakes, NJ). HRP conjugated secondary antibodies were obtained from Pierce (Rockford, IL). For detection of eNOS dimers, we sought to minimize artifacts due to ex vivo disruption of eNOS dimers by performing low temperature (four degrees celcius) polyacrylamide electrophoresis as previously described\textsuperscript{7}. Pilot experiments demonstrated that incubation of the samples at 4, 25, and 37 degrees in the presence of SDS and 2.5% \(\beta\)-mercaptoethanol for up to 2 hours was not sufficient to denature the proteins in the arterial lysate, and eNOS protein did not migrate according to size on SDS-PAGE. When the protein lysates were heated to 65 degrees for 30 minutes in the presence of SDS and 2.5% \(\beta\)-mercaptoethanol, eNOS protein migrated according to its expected molecular weight. Although the heating procedure may have led to an underestimation of the absolute amount of eNOS dimers, all samples from both diet groups were processed simultaneously under the same conditions, and the relative differences between the diet groups persisted in all the experiments.

Nitrotyrosine immunohistochemistry: Sections from mice (\(n=3\) from each diet group) were fixed with acetone and blocked with 8% goat serum in PBS for 1 hour. Sections were incubated with 1:100 dilution of rabbit anti-nitrotyrosine antibody (Upstate Technologies) in 4% goat serum overnight at 4 degrees. After washing, alexa 488 conjugated anti-rabbit antibody (Molecular Probes) was applied for 30 minutes, sections were washed and incubated with hoechst dye33258
(Molecular Probes) and coverslipped. Fluorescence images were acquired using fixed exposure times using a CCD camera attached to a Zeiss Axiovert inverted microscope. Negative and positive control sections were preincubated with 3-nitrotyrosine (Sigma), or a peroxynitrite solution (Upstate), respectively, to assure specificity of staining.

Statistics: Data are represented as mean±standard error of the mean (sem). Significance was assessed by two-tailed Student’s t-test for parametric data at p<0.05. Bonferroni post tests were utilized after 2 way analysis of variance (ANOVA) to detect differences between groups. Statistical tests were performed using Prism software (GraphPad).
### Online Table I

<table>
<thead>
<tr>
<th>Diet</th>
<th>Period of feeding</th>
<th>Weight</th>
<th>Leptin</th>
<th>Glucose</th>
<th>Insulin</th>
<th>TC</th>
<th>TG</th>
<th>FFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>chow</td>
<td>9 weeks</td>
<td>32±1(17)</td>
<td>4±1(10)</td>
<td>135±7(10)</td>
<td>0.28±0.04(10)</td>
<td>79±4(7)</td>
<td>46±2(7)</td>
<td>0.59±0.04(10)</td>
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<tr>
<td>high fat</td>
<td>9 weeks</td>
<td>39±1(30)*</td>
<td>56±5(20)*</td>
<td>226±9(20)*</td>
<td>2.5±0.36(25)*</td>
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