Neointimal Formation After Endovascular Arterial Injury Is Markedly Attenuated in db/db Mice

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Objective—A diabetic mouse model of accelerated neointimal formation would be a useful tool to understand the increased incidence of restenosis in patients with diabetes.

Methods and Results—Femoral artery endoluminal wire injury was performed in diabetic insulin 2 Akita (ins2Akita) and leptin receptor db/db (leprdb/db) mutant mice. Neointima size in ins2Akita mouse arteries was unchanged compared with nondiabetic wild-type littermates. Although Ki67 labeling demonstrated similar rates of replication in the neointima of leprdb/db mouse arteries, neointimal formation in leprdb/db mice was surprisingly reduced by ~90% compared with nondiabetic lepr+/+ mice. Four hours after arterial injury, medial smooth muscle cell death was diminished in leprdb/db arteries, suggesting that the initial response to arterial injury was altered in leprdb/db mice.

Conclusions—These studies highlight a differential response to arterial injury in leprdb/db mice and suggest a potential role for leptin in the regulation of neointimal formation in response to arterial injury. (Arterioscler Thromb Vasc Biol. 2003; 23:●●●●●●●●.)

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Numerous clinical trial and observational data have indicated that diabetic patients have a higher incidence of restenosis after percutaneous coronary transluminal angioplasty and stent implantation compared with patients without diabetes.1–5 Serial intravascular ultrasound studies suggest that excessive neointimal hyperplasia is responsible for accelerated restenosis after angioplasty and coronary stent placement in patients with diabetes and impaired glucose tolerance.6,7 However, the relationships between the metabolic abnormalities present in diabetes and the cellular and molecular processes involved in restenosis are poorly understood.

A diabetic mouse model of accelerated neointimal formation would be a useful tool to understand the increased incidence of restenosis in patients with diabetes. Balloon angioplasty is technically challenging in the mouse because of the small vessel size, and a variety of wire injury models exist.8–14 Recent work has demonstrated that reproducible arterial injury can be performed in the mouse femoral artery using an angioplasty guidewire.8,15 We chose to study neointimal formation after bilateral femoral arterial injury in the diabetic, nonobese insulin 2 Akita (ins2Akita) and diabetic, obese leptin receptor db/db (leprdb/db) mouse strains. Ins2Akita mice have a spontaneous dominant mutation in the insulin 2 gene, resulting in defective proinsulin chain folding, reduced β cell mass, hypoinsulinemia, and hyperglycemia.16 The ins2Akita mouse serves as a type I model of diabetes that does not require administration of a chemical diabetogen such as streptozotocin. leprdb/db mice have a mutation in the leptin receptor gene, resulting in a mutant protein lacking the cytoplasmic domain required for intracellular signaling.17 The leprdb/db model has many of the metabolic abnormalities (albeit more severe) noted in human obese diabetic subjects, such as obesity, hyperglycemia, and insulin resistance.17 We initially predicted that hyperglycemia or hyperinsulinemia would accelerate neointimal formation in response to arterial injury. Hyperglycemia per se did not alter neointimal formation in the ins2Akita mice, and neointimal formation was nearly absent in leprdb/db mice. Analysis of the early response to arterial injury in leprdb/db mice revealed substantial decreases in medial smooth muscle cell death. The near absence of neointimal formation in the leprdb/db mouse suggests a new potential role for leptin in the response to arterial injury.

Methods

Mice
C57Bl/6 ins2Akita heterozygotes were obtained from Jackson Laboratory (stock No. 3548) and were bred with C57BL/6 mice to obtain C57Bl/6 ins2Akita heterozygotes and wild-type littermate controls for use in arterial injury experiments. A polymerase chain reaction (PCR) assay16,18 to identify the ins2Akita mutation was first used to identify C57Bl/6 ins2Akita heterozygotes. In all cases, a positive genotype correlated with a fasting blood glucose >300 mg/dL.

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Therefore, a fasting glucose >300 mg/dL was subsequently used to identify ins2<sup>−/−</sup> mice. C57BL/6 mice were identified as mice having a fasting blood glucose <200 mg/dL. C57BLKS/Lepr<sup>−/−</sup> breeding pairs were obtained from Jackson Laboratory (BKS.Cg-Ins2<sup>−/−</sup>Lepr<sup>−/−</sup>leprdb; stock No. 000642). The genotype of the mice was confirmed using a PCR/restriction-length polymorphism assay that can identify the db point mutation at the Lepr locus. Type 2 phenotypes based on coat color and body habits agreed with PCR genotype in all cases. Therefore, Lepr<sup>−/+</sup> and Lepr<sup>−/−</sup> mice were subsequently identified by phenotype. The incorporation of the misty (m) gene tightly linked to Lepr<sup>−/−</sup> of sex- and age-matched Akita mice compared with PCR genotype in all cases. Therefore, Lepr<sup>−/−</sup> and Lepr<sup>−/−</sup> mice were identified by their phenotypic appearance at 8 weeks of age, as previously described (http://jaxmice.jax.org/jax-mice/guide/jaxmicedb.cgi?obotype=pricedetail&stock=000642&dest=N).

Briefly, body habits and coat color were used to phenotype the mice. Lepr<sup>−/−</sup> (genotype: m, lepr<sub>2</sub>/m, lepr<sub>2</sub>+) mice were gray and lean; Lepr<sup>−/+</sup> (genotype m, +/+, lepr<sub>2</sub>) were black and lean; and Lepr<sup>−/−</sup> (genotype: +, lepr<sub>2</sub>/+, lepr<sub>2</sub>) were black and obese. All procedures were approved by the Institutional Animal Care and Use Committee, consistent with the Guide for the Care and Use of Laboratory Animals.

Arterial Injury

Bilateral femoral artery procedure was performed as previously described<sup>8–13</sup> with a few modifications. The arteriotomy was made in the superficial branch of the femoral artery, and a 0.3-mm angioplasty guidewire was used to denude and dilate the artery. The wire was removed and the superficial femoral artery was ligated, diverting blood flow down the deep femoral branch. Mice were given free access to food and water before and after arterial injury, and arterial specimens were obtained as previously described.<sup>8–15</sup> Four-month-old female ins2<sup>−/−</sup> and ins2<sup>−/+</sup> were euthanized 4 weeks after arterial injury. Despite setting up large numbers of synchronous matings of lepr<sup>−/−</sup> mice, we were unable to obtain sufficient numbers of sex- and age-matched Lepr<sup>−/−</sup> and Lepr<sup>−/−</sup> mice for euthanasia at 2 different time points after arterial injury. Therefore, we arbitrarily euthanized female Lepr<sup>−/−</sup> mice at 4 weeks and male Lepr<sup>−/−</sup> mice at 2 weeks after arterial injury. Additional sections were obtained for immunohistochemical staining of platelets (rabbit anti-mouse thrombocyte, Cederlane Laboratories, Ontario, Canada) and smooth muscle cells (α-actin; Dako, JA4). The area occupied by immunostained cells (expressed as ratio of area of immunostained cells per arteriole circumference for platelets or area of immunostained cells per intimal area for α-actin) was quantified for each injured artery. Sections were then processed for terminal deoxynucleotidyl TUNEL staining using a commercially available kit according to the manufacturer’s instructions (fluorescein in situ cell death detection kit, Roche). Sections were also incubated with DAPI (Molecular Probes) to identify nuclei. Fluorescein (reflecting TUNEL-positive cells) and DAPI epifluorescence from all sections were viewed and simultaneously digitized using fixed exposure times on an epifluorescence microscope. Ki67 (Novocastra) and active Caspase-3 (BD Pharmingen) staining were visualized with a Vectastain ABC Elite kit, +/+ and DAB substrate (Vector Laboratories). Comparisons between genotypes were performed using the Student’s t test using Prism software (Graph Pad). Analysis of males and females were performed separately.

Biochemical Measurements

At the time of euthanasia, mice were fasted for 6 hours and blood was obtained by ventricular puncture used to obtain plasma for lipoprotein fractions as previously described.<sup>20</sup> Plasma was isolated from tail vein blood samples from fasted mice for measurements of plasma glucose (Sigma) and insulin (Linco).

Results

We determined whether the presence of type 1 or type 2 diabetes would promote neointimal formation after femoral artery endovascular injury. ins2<sup>−/−</sup> mutant mice were used as a model of type 1 diabetes. Ins2<sup>−/−</sup> mice had normal body weight (27±1 versus 26±2 g; n=8 to 9 per group; P=NS) and were hyperglycemic (411±48 versus 150±26 mg/dL, n=7 to 9 per group, P<0.0001) compared with nondiabetic littermate controls. The presence of type 1 diabetes did not increase neointimal formation in the ins2<sup>−/−</sup> mouse model (Figures 1A and 1B). No significant differences in intimal area, medial area, and intimal to medial ratio (I/M) were noted after arterial injury in ins2<sup>−/−</sup> mice compared with nondiabetic wild-type controls (Figure 1C).

Femoral artery endovascular injury was also performed in Lepr<sup>−/−</sup> mutant mice, a model of type 2 diabetes. As previously reported,<sup>21,22</sup> Lepr<sup>−/−</sup> mice were obese, hyperglycemic, hyperinsulinemic, and hypercholesterolemic (Table 1 and Figure 1, available online at http://atvb.ahajournals.org). After arterial injury, the mean intimal area and I/M in male Lepr<sup>−/−</sup> mice were reduced by 88% and 85%, respectively, compared with male Lepr<sup>−/+</sup> mice (Figures 2A and 2C). No differences in medial area were noted in male mice after arterial injury (Figure 2B). In female Lepr<sup>−/−</sup> mice, intimal area and I/M were reduced by 95% and 93%, respectively (Figures 2D and 2F). In contrast to male mice, there was also a small but significant decrease in medial area in female Lepr<sup>−/−</sup> mice (Figure 2E). Histological examination of cross sections through the femoral artery from Lepr<sup>−/+</sup> mice revealed the presence of a neointima (Figures 3C and 3D). Many of these neointimal cells stained positive with anti-smooth muscle cell α-actin antibody, indicating a smooth muscle cell phenotype (data not shown). In contrast, a neointima was absent in nearly all sections from injured arteries from Lepr<sup>−/−</sup> mice (Figures 3E and 3F), and arteries appeared very similar to sham-injured arteries (Figures 3A and 3B). Ki67 labeling was performed on arterial sections from Lepr<sup>−/+</sup> mice and on the limited number of femoral artery sections showing neointima in Lepr<sup>−/−</sup> mice (Figure 4). The percentage of intimal nuclei labeled with Ki67 was not different in the neointima from Lepr<sup>−/−</sup> compared with Lepr<sup>−/−</sup> mice (32±7.4, n=6, versus 35±18, n=2; P=NS).

Lepr<sup>−/+</sup> and Lepr<sup>−/−</sup> mice were euthanized 4 hours after bilateral femoral artery injury to identify potential differences in the initial response to arterial injury that may be responsible for the inhibition of neointima in Lepr<sup>−/−</sup> mice. Analysis of sham-injured arteries did not reveal any significant differences in lumen (0.028±0.007 versus 0.037±0.002 mm<sup>2</sup>, P=NS) and vessel (0.040±0.009 versus 0.048±0.001 mm<sup>2</sup>, P=NS) area in the femoral arteries of Lepr<sup>−/+</sup> mice compared with Lepr<sup>−/−</sup> mice. There were also no differences in the area occupied by adherent platelets (0.001±0.0006 versus 0.0009±0.0004 mm<sup>2</sup>, n=5, P=NS; see Figure 5). Alterations in the arterial media were noted in Lepr<sup>−/+</sup> mice compared with Lepr<sup>−/−</sup> mice. The media from injured femoral arteries from Lepr<sup>−/+</sup> mice contained multiple areas devoid of cell nuclei (Figures 6C and 6D), with a decrease in the area occupied by α-actin–positive cells compared with Lepr<sup>−/−</sup> and sham-injured Lepr<sup>−/+</sup> mice (Figure 6G). In contrast, the media of injured femoral arteries harvested from Lepr<sup>−/−</sup> mice (Figures 6E and 6F) appeared similar to the media from sham-injured femoral arteries (Figures 6A and 6B), with no change in the area of α-actin–immunostained cells (Figure...
6G). These findings suggested that mechanical injury of the femoral artery resulted in medial smooth muscle cell death in lepr/H/H11001 mice but not in lepr/db/db mice. To determine whether the difference in smooth muscle cell nuclear dropout and α-actin staining was attributable to mechanical injury–induced apoptosis, TUNEL staining was performed on sections derived from mice euthanized 4 hours after injury. Frequent TUNEL-positive smooth muscle cells were noted in sections derived from lepr+/+ femoral arteries (Figure IIA, available online), but TUNEL-positive cells were rarely seen in arteries harvested from lepr/db/db mice (Figure IIB, available online). The area occupied by TUNEL-positive cells per unit area of

Figure 1. Representative photomicrographs (×100) of combined Masson elastin–stained femoral artery sections and morphometric measurements from C57BL/6 wild-type and ins2/Akita mice 4 weeks after injury. Neointima was present in sections from both C57BL/6 wild-type mice (A) and ins2/Akita mice (B). Morphometric measurements revealed no significant difference in intimal area, media area, or I/M ratio between the 2 groups of mice (C).

Figure 2. Morphometric measurements of cross sections of injured femoral arteries from lepr+/+ and lepr/db/db mice. Measurements were obtained from male (A, B, and C) and female (D, E, and F) mice 2 and 4 weeks after injury, respectively. Bars denote the mean±SEM of intimal area (A and D), medial area (B and E), and I/M ratio (C and F). The number of arteries injured is denoted in parentheses.
media was markedly reduced in lepr<sup>db/db</sup> mice compared with lepr<sup>+/+</sup> mice (Figure IIC). Staining for activated caspase-3 (Figure III, available online) did not identify apoptotic cells in the media 4 hours after arterial injury, suggesting that the TUNEL staining highlighted cell damage attributable to mechanical injury to cells and likely was not identifying cellular apoptosis. These results indicate that the differences in the extent of smooth muscle cell death 4 hours after arterial injury were related to reductions in mechanical injury–mediated smooth muscle cell necrosis in lepr<sup>db/db</sup> mice.

**Discussion**

Endovascular femoral artery injury was performed in mouse models of type 1 and 2 diabetes mellitus in an effort to create a mouse model in which diabetes accelerates the response to arterial injury. Neointimal size was unchanged after femoral artery injury of ins2<sup>−/−</sup> mice, a model of type 1 diabetes. Similar to our results, type 1 diabetes did not alter neointimal formation in streptozotocin-treated Sprague-Dawley rats. In contrast, neointimal formation in lepr<sup>db/db</sup> mice, a model of
Figure 5. Representative photomicrographs (×100) of femoral artery sections from lepr<sup>+/+</sup> and lepr<sup>db/db</sup> mice 4 hours after arterial injury. Anti-thrombocyte antibody staining revealed platelet adherence to the injured arterial wall in femoral arteries from lepr<sup>+/+</sup> (A) and lepr<sup>db/db</sup> (B) mice.

Figure 6. Immunohistochemical staining for smooth muscle α-actin in femoral artery sections obtained 4 hours after arterial injury. A significant loss of medial α-actin-positive smooth muscle cells was noted in injured arterial sections from lepr<sup>+/+</sup> mice (C and D). The decrease in α-actin-immunostained medial cells was not observed in arterial sections from lepr<sup>db/db</sup> mice (E and F) and sham-injured lepr<sup>+/+</sup> mice (A and B). Quantification of the percentage of the media occupied by α-actin-positive cells is displayed in G. B, D, and F are high-magnification (×400) images of the boxed regions shown on low magnification (×100) images A, C, and E.
type II diabetes, was markedly attenuated. This seemingly paradoxical effect is consistent with previous reports in leptin-dependent mouse models demonstrating impaired responses in several models of tissue injury.24–28 In response to FeCl₃ or photochemical-induced injury of the carotid arteries, platelet adherence and time of vessel occlusion is delayed in both leptindependent and leptin-independent mice.27,28 Leptin-deficient mice have a point mutation in the leptin gene, resulting in absent leptin levels, obesity, diabetes, and a phenotype nearly identical to the db/db mouse.29 Immunoactive platelet deposition at 4 hours was unchanged after endovascular wire injury of leptin-deficient mice in the present study. Variations in the type of injury model and the cellular events comprising the response to injury may underlie the differences in platelet adhesion among the studies. In addition, thrombosis is an uncommon finding in the wire injury model but is the end point in the FeCl₃, and photochemical injury models. Nevertheless, the near absence of neointima in leptin-deficient mice in the present study provides additional evidence for an attenuated vascular response to arterial injury in leptin-deficient mice. Preservation of α-actin–positive medial smooth muscle cells 4 hours after arterial injury indicates that differences exist in the initial response to arterial injury in leptin-deficient mice. No significant differences in lumen size were observed in sham-injured leptin-deficient and leptin-receptor mutant mice. Alterations in arterial caliber, tone, or compliance in the femoral artery of leptin-deficient mice may have affected the magnitude of the mechanical force imparted to the femoral artery by the wire, resulting in inhibition of neointimal formation. Leptin has been shown to increase sympathetic nerve activity and arterial blood pressure in experimental models.30,31 Although arterial blood pressure in leptin-receptor mutant mice has been reported to be decreased compared with wild-type controls,32 other studies have documented no change in blood pressure in both leptin-deficient and leptin-receptor mutant mice.30,33 Theoretically, a decrease in sympathetic nervous system activity or a decrease in arterial blood pressure could have had inhibitory effects on the response to arterial injury in leptin-deficient mice in the present study. However, arterial rings isolated from leptin-deficient and leptin-receptor mutant mice have enhanced contractile responses to noradrenaline and diminished relaxation to acetylcholine.35,36 The enhanced contractile responses would be expected to increase the mechanical force of injury and do not account for the diminished response to arterial injury in leptin-deficient mice.

Medial smooth muscle cell death after arterial injury has been observed in numerous animal models of endovascular injury.37–39 The extent of TUNEL–positive staining was reduced in leptin-deficient mice compared with leptin-receptor mutant mice, but we were unable to document smooth muscle cell apoptosis by staining for activated caspase-3 at 4 hours after arterial injury. However, it is likely that both medial smooth muscle cell necrosis and apoptosis play a role in medial smooth muscle cell death after endovascular injury, because other reports have documented chromatin condensation on transmission electron microscopy and DNA fragmentation in medial smooth muscle cells after mechanical endovascular injury in the rat and the mouse.37–39 It is possible that decreases or delays in medial smooth muscle cell death may have affected the elaboration of chemotactic factors important for smooth muscle cell migration into the intima. At 2 weeks after arterial injury, Ki67 staining was unchanged in the neointima of leptin-deficient mice. These data suggest that the marked inhibition of neointimal formation in leptin-deficient mice was not a direct result of diminished cellular proliferation but was more likely attributable to inhibition of events important in the initiation of smooth muscle migration in response to arterial injury.

The mechanism for the paradoxical reduction of neointimal formation in leptin-deficient mice may be difficult to elucidate without the use of additional mutant mouse models. It is intriguing to speculate that inhibition of leptin signaling at the level of the vessel may have directly or indirectly impaired the response to arterial injury in the leptin-deficient mice. Leptin administration just before FeCl₃, or photochemical-induced carotid injury restores the time to thrombosis (to that observed in wild-type mice) in mice lacking leptin (leptin-deficient) but not in mice with defective leptin signaling (leptin-receptor mutant).27,28 Arterial injury studies in the obese Zucker rat with a mutation in the leptin receptor (fa/fa) have not been consistent. One study demonstrated a 2-fold increase in neointimal formation in balloon-injured arteries;33 however, this was not reproduced in a subsequent study.40 In addition to the central role of leptin in the regulation of food intake and energy balance,41 leptin seems to have numerous effects on vascular cells. Experimental studies have documented effects of leptin on vascular tone,42,43 endothelial reactive oxygen species generation,44 angiogenesis,45 proliferation of cultured vascular smooth muscle cells,46 platelet aggregation,47 and HDL metabolism.48 Elevation in HDL cholesterol in leptin-deficient mice may have had an inhibitory effect on neointimal formation. Previous studies have demonstrated that human apoA-I exerts a protective effect on neointimal formation in injured mouse carotid arteries in hyperlipidemic mice.48,49 However, it is unclear whether this protective effect of HDL on neointimal formation occurs after endovascular injury in humans, because clinical studies have not clearly established low HDL as a predictor of atherosclerosis after revascularization procedures.50,51

The understanding of the relationships among metabolic parameters and restenosis may lead to the identification of therapeutic agents that have salutary effects on both metabolism and restenosis. Thiazolidinediones, insulin-sensitizing agents used for glycemic control in humans, have been effective in reducing intimal formation in Zucker fatty and Sprague-Dawley rats52,53 and in one small human trial.54 Additional arterial injury studies in leptin-dependent (such as leptin-deficient) and leptin-independent mouse models of type 2 diabetes should provide additional insight into whether the attenuated response to arterial injury is specific for leptin receptor mutant mice. It remains to be seen whether the presence of insulin resistance and diabetes will increase the response to arterial injury in the mouse.

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