Recovery from hind limb ischemia is less effective in type 2 than in type 1 diabetic mice: Roles of endothelial nitric oxide synthase and endothelial progenitor cells

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Objective: **We sought to directly compare the effects of type 1 and type 2 diabetes on postischemic neovascularization and evaluate the mechanisms underlying differences between these groups. We tested the hypothesis that type 2 diabetic mice have a greater reduction in endothelial nitric oxide synthase (eNOS) expression, a greater increase in oxidative stress, and reduced arteriogenesis and angiogenesis, resulting in less complete blood flow recovery than type 1 diabetic mice after induction of hind limb ischemia.**

Methods: **Hind limb ischemia was generated by femoral artery excision in streptozotocin-treated mice (model of type 1 diabetes), in *Lepr$^{db/db}$* mice (model of type 2 diabetes), and in control (C57BL/6) mice. Dependent variables included eNOS expression and markers of arteriogenesis, angiogenesis, and oxidative stress.**

Results: **Postischemia recovery of hind limb perfusion was significantly less in type 2 than in type 1 diabetic mice; however, neither group demonstrated a significant increase in collateral artery diameter or collateral artery angioscore in the ischemic hind limb. The capillary/myofiber ratio in the gastrocnemius muscle decreased in response to ischemia in control or type 1 diabetic mice but remained the same in type 2 diabetic mice. Gastrocnemius muscle eNOS expression was lower in type 1 and 2 diabetic mice than in control mice. This expression decreased after induction of ischemia in type 2 but not in type 1 diabetic mice. The percentage of endothelial progenitor cells (EPC) in the peripheral blood failed to increase in either diabetic group after induction of ischemia, whereas this variable significantly increased in the control group in response to ischemia. EPC eNOS expression decreased after induction of ischemia in type 1 but not in type 2 diabetic mice. EPC nitrotyrosine accumulation increased after induction of ischemia in type 2 but not in type 1 diabetic mice. EPC migration in response to vascular endothelial growth factor was reduced in type 1 and type 2 diabetic mice vs control mice. EPC incorporation into tubular structures was less effective in type 2 diabetic mice. Extensive fatty infiltration was present in ischemic muscle of type 2 but not in type 1 diabetic mice.**

Conclusion: **Type 2 diabetic mice displayed a significantly less effective response to hind limb ischemia than type 1 diabetic mice. (J Vasc Surg 2009;50:1412-22.)**

Clinical Relevance: **Diabetes is important in the pathogenesis of peripheral artery disease. The present study demonstrates that the vascular response to acute hind limb ischemia is dependent on the type of diabetes present. Type 2 diabetic mice (*Lepr$^{db/db}$*) demonstrated significantly less effective blood flow recovery than type 1 diabetic mice (streptozotocin-induced). Moreover, the differences between diabetic groups appeared contingent, at least in part, on differences in endothelial nitric oxide, oxidant stress, and endothelial progenitor cell function between the two diabetic groups. Although direct extrapolation of animal data to the human experience must be made with caution, these findings indicate that the type of diabetes present, and not just the presence of diabetes per se, may be important in the initiation of progression of peripheral artery disease.**

Vascular dysfunction underlies many of the complications of diabetes mellitus, including peripheral artery disease.[1] However, the basis for this dysfunction is dissimilar in type 1 and type 2 diabetes. Type 1 diabetes is characterized by hypoinsulinemia and profound hyperglycemia. The latter circumstance is particularly injurious to endothelial cells, which lack the capacity to regulate glucose influx.[1] Uncontrolled glucose influx results in activation of protein kinase C and the accumulation of advanced glycation end products and oxidants within the endothelial cell, processes that instigate endothelial dysfunction.[1] In contrast, type 2 diabetes is characterized by insulin resistance, moderate hyperglycemia, and an

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atherogenic dyslipidemia; consequently, the vascular dysfunction in type 2 diabetes reflects the effects of hypercholesterolemia and not just hyperglycemia on the endothelium.[1-3]

This distinction may be relevant in the design of treatments for diabetic vascular disease, such as peripheral artery disease. An important gap in knowledge, however, is the lack of a direct comparison between the effects of type 1 and 2 diabetes on the response to vascular injury, that is, an assessment that applies the identical experimental paradigm to both types of diabetes.

Assessment of hind limb hemodynamics after ligation or excision of the common femoral artery is a widely used approach to study postischemic perfusion recovery and neovascularization.[4] By this means, Tamarat et al[5] reported significant compromise in postischemic hind limb flow recovery in streptozotocin-induced type 1 diabetic mice, whereas Emaueli et al[6] described similar findings in the *Lepr$^{db/db}$* mouse model of type 2 diabetes. Methodologic differences between these studies, however, preclude their use as instruments to directly compare neovascularization responses between types 1 and 2 diabetes.

Bone marrow-derived endothelial progenitor cells (EPC) are critical participants in postischemic neovascularization.[7-9] EPC are mobilized from bone marrow and home to sites of vascular injury, where they contribute to arteriogenesis within existing collateral arteries and to angiogenesis within capillary networks. Dysfunction in EPC mobilization and function has been reported in type 1 and 2 diabetes.[10-13] Deficiencies in the generation of endothelial nitric oxide synthase (eNOS)-derived nitric oxide[14,15] and an increased level of oxidant stress[16,17] have been proposed as mechanisms responsible for EPC dysfunction in diabetes. Again, however, direct comparison of these circumstances between type 1 and 2 diabetes has not been reported.

The goal of this study was to compare the effects of type 1 vs type 2 diabetes on blood flow recovery and neovascularization after induction of severe hind limb ischemia using streptozotocin-induced hypoinsulinemia as a model of type 1 diabetes and the *Lepr$^{db/db}$* mouse as a model of type 2 diabetes. We tested the hypothesis that type 2 diabetic mice have a greater reduction in eNOS expression, a greater increase in oxidative stress, reduced arteriogenesis, and angiogenesis resulting in less complete blood flow recovery than type 1 diabetic mice after induction of hind limb ischemia.

MATERIALS AND METHODS

Experimental animals. The mice used in this study were obtained from Jackson Laboratories (Bar Harbor, Me), were housed in an environmentally controlled room, and were fed standard chow and water before and during the course of study. The care of mice complied with the National Research Council *Guide for the Care and Use of Laboratory Animals*. All protocols were approved by the Committees on Animal Research at the University of California, San Francisco, and the University of Massachusetts Medical School.

Antibodies and reagents. Streptozotocin, Dil-Ac-LDL, Histopaque 1083, Matrigel, and the monoclonal antibody for smooth muscle actin were obtained from Sigma-Aldrich (St. Louis, Mo). Monoclonal antibodies for CD31, eNOS, and nitrotyrosine were obtained from BD Bioscience (San Diego, Calif). Monoclonal antibodies for CD34, Flk-1, and CD133 were obtained from eBioscience (San Jose, Calif). Vascular endothelial growth factor (VEGF) was obtained from R&D Systems (Minneapolis, Minn). Oil Red O was obtained from Poly Scientific (Bay Shore, NY).

Experimental animals and procedures. C57BL/6 mice were used as the control group. Type 1 diabetes was generated in 8-week-old C57BL/6 mice by an intraperitoneal injection of streptozotocin (50 mg/kg daily for 5 days). Streptozotocin generates significant, predictable, and reproducible hypoinsulinemia and profound hyperglycemia within 1 week after the onset of treatment. The *Lepr$^{db/db}$* (*db/db*) mouse, generated on a C57BL/6 background, was used as a model of type 2 diabetes. The *db/db* mice are deficient in the Ob-Rb leptin receptor, critical in regulation of satiety and hence food intake; consequently, *db/db* mice become significantly obese. The *db/db* mice fully express a type 2 diabetic phenotype of insulin resistance, moderate hyperglycemia, and dyslipidemia by 2 months of age.[18,19]

Hind limb ischemia was induced as previously described.[20] Briefly, mice were anesthetized using 2% isoflurane, and the left femoral artery and its associated branches were isolated, ligated, and excised. The contralateral hind limb served as an internal control within each mouse.

Measurement of hind limb blood flow. A laser Doppler perfusion imager (Moor Instruments Ltd, Devon, United Kingdom) was used to estimate dermal blood flow in the calf and foot. Hind limb fur was removed by depilatory cream, and studies were conducted under 1.5% isoflurane anesthesia. Mice were studied while on a heated surface (37°C) and in a darkened room to minimize the effects of ambient light and temperature on measurements. Blood flow was expressed as a ratio of the ischemic and nonischemic hind limbs.

Measurement of collateral artery enlargement and number. At 28 days after induction of hind limb ischemia, mice were anesthetized with 1.5% isoflurane, and a contrast medium was injected into the abdominal aorta through a catheter. Images were taken by a digital X-ray transducer. Two observers independently scored the images by counting the number of vessels that crossed a standardized grid overlying the image. The number of vessels was divided by the lines of the grid in the area of interest to produce an angioscore. It was not possible to completely blind these measurements because the obesity of the *db/db* type 2 diabetic mice was obvious on the radiographs.

Measurement of collateral artery diameter. Thigh muscle was harvested 28 days after induction of ischemia and frozen at −80°C in OCT. Cryosections (10 μm) were prepared and incubated with CD31 and smooth muscle actin antibodies for the identification of endothelial cells

and smooth muscle cells, respectively, to identify collateral arteries. Collateral artery diameter was measured using precalibrated microscope scale bars. Three sections were reviewed per animal, and collateral diameters were quantified in five randomly selected low-power (original magnification ×200) fields per section. The mean value of these measurements was taken as a single data point for each animal.

Measurement of capillary density. Gastrocnemius muscle was harvested 28 days after induction of ischemia and frozen at −80°C in OCT. Cryosections (10 μm) were prepared and incubated with CD31 antibody to identify endothelial cells. Capillary density was expressed as the ratio of $CD31^+$ cells to myofibers. This measurement was determined in five randomly selected low-power (original magnification ×200) fields from each animal, and the average value was used as a single data point for each mouse.

Assessment of muscle histology. Tibialis anterior and gastrocnemius muscles were harvested on day 28 after induction of ischemia, weighed, and frozen at −80°C in OCT. Cryosections (10 μm) were prepared and stained with hematoxylin and eosin. To identify adipocytes, some cryosections were fixed in 10% formalin for 10 minutes, stained with Oil Red O at 60°C for 8 minutes and then counterstained briefly with eosin. Five low-power (original magnification ×200) fields were randomly selected on each slide, and the degree of necrosis and adipocyte infiltration noted in each.

Measurement of eNOS and nitrotyrosine. Muscle tissue was harvested 7 days after induction of ischemia, and EPCs were obtained from bone marrow or peripheral blood 7 days after induction of ischemia. Tissue (muscle or EPC) was homogenized in buffer (50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [pH 7.5], 150 mM magnesium chloride, 1 mM ethylenediaminetetraacetic acid, 100 mM sodium chloride, 1% NP40). Protein extracts underwent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), were transferred to pure nitrocellulose membranes, and were probed with antibodies to eNOS, nitrotyrosine, or α-tubulin. Enhanced chemiluminescence detection was used to visualize bands. Band intensity was determined by densitometry. Densitometry data for the eNOS and nitrotyrosine bands were expressed as a ratio to the α-tubulin band.

Quantitation of EPC in bone marrow and peripheral blood. Samples of bone marrow and peripheral blood were obtained 7 days after induction of ischemia. Mononuclear cells were isolated by gradient centrifugation using Histopaque 1083. Mononuclear cells (10^6) were incubated for 10 minutes with monoclonal antibodies for CD34, Flk-1, and CD133. Isotype antibodies were used as negative controls. Cells were analyzed on a fluorescence-activated cell sorter (FACS) Calibur Flow Cytometer (BD Bioscience, San Jose, Calif). Each analysis consisted of 500,000 events. Data were analyzed with FlowJo software (Tree Star, Inc, Ashland, Ore).

Cultivation of EPCs. Bone marrow and peripheral blood were harvested 7 days after induction of ischemia. Mononuclear cells were isolated by gradient centrifugation and cultured on fibronectin-coated plates in endothelial cell basal medium supplemented with endothelial growth medium. After 3 days, nonadherent cells were removed by washing the plates with phosphate-buffered saline. Adherent cells were used for in vitro functional analysis.

Measurement of EPC migration. In vitro migration was evaluated in bone marrow-derived EPCs with the use of a modified Boyden chamber. Cell suspensions (5×10^4 cells/well) were placed in the upper chamber, and the lower chamber was filled with medium (control) or medium containing human recombinant VEGF (50 ng/mL). The chamber was incubated for 16 hours at 37°C. Migration activity was evaluated by counting the number of cells on the lower chamber surface. This count was replicated in three high-power (original magnification ×400) fields per chamber. The mean count was determined and taken as an individual data point for each assay.

Measurement of EPC tubular incorporation. Matrigel was thawed and placed in four-well glass slides at room temperature for 30 minutes to allow solidification. Dil-Ac-LDL-labeled EPCs (2×10^4) were co-plated with 4×10^4 human umbilical vein endothelial cells and incubated at 37°C for 12 hours. The incorporation of EPCs in tubules was determined in five random high-power (original magnification ×400) fields. The mean was determined and taken as an individual data point for each assay.

Statistical analysis. Data were analyzed by one-way analysis of variance followed by post hoc Student-Newman-Keuls test. Statistical significance was accepted at the 95% level ($P < .05$) for all analyses. Mean data are presented with the standard deviation.

RESULTS

Blood glucose and body weight. The blood glucose concentration was measured in all mice before the study began to confirm a diabetic phenotype. The fasting blood glucose was 460 ± 36 mg/dL in treptozotocin-treated type 1 diabetic mice, 264 ± 14 mg/dL ($P < .05$) in *db/db* type 2 mice, and 112 ± 12 mg/dL in control mice ($P < .05$ vs type 1 and type 2 diabetic mice). Plasma insulin and serum lipids were not measured because streptozotocin induces profound hypoinsulinemia, and the *db/db* mutation results in insulin resistance and an atherogenic dyslipidemia.[18,19] Type 2 diabetic mice weighed 52 ± 6 g compared with 22 ± 1 g for type 1 diabetic mice and 24 ± 2 g for control mice ($P < .05$ for type 2 diabetic mice vs type 1 diabetic mice or control mice).

Effect of ischemia on arteriogenesis. Blood flow recovery after induction of severe hind limb ischemia was significantly less in type 1 and type 2 diabetic mice than in control mice. In the calf, these differences became significant on day 14 after induction of ischemia; whereas in the foot, significance was attained on day 21 (Fig 1). Blood flow recovery was thus less complete in type 2 than in type 1 diabetic mice: maximal recovery reached 22% and 43% in the foot and calf, respectively, in type 2 diabetic mice compared with 42% and 63% in the foot and calf, respectively, in type 1 diabetic mice ($P < .05$).

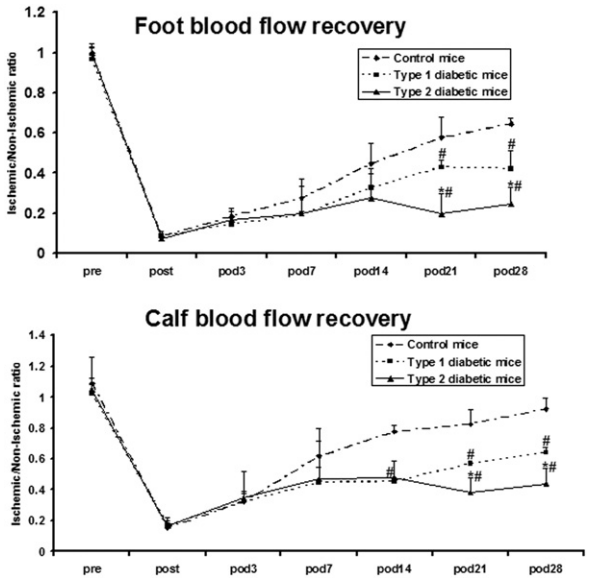

Fig 1. Dermal blood flow in the **(top)** foot and **(bottom)** calf was measured in the same animal at multiple times before and after induction of ischemia (n = 5 animals in each group). Flow data represent the ratio of the ischemic to nonischemic hind limb. Mean data are presented with the standard deviation *(error bars)*. *Pre,* Preoperative; *Post,* immediately postoperative, *POD,* postoperative day. $^{\#}P < .05$ for type 1 or type 2 diabetic mice vs control mice and $^{*}P < .05$ for type 2 vs type 1 diabetic mice.

Type 2 diabetic mice had collateral arteries of significantly smaller diameter in the nonischemic hind limb than type 1 diabetic or control mice (Fig 2, *A* and *B*). Collateral artery diameter was significantly greater in the ischemic than nonischemic hind limb in control mice. In contrast, the collateral artery diameter was similar in the ischemic and nonischemic hind limb of type 1 and 2 diabetic mice. Angiography revealed the presence of a well-developed collateral artery network in the ischemic thigh of control mice (Fig 2, *C* and *D*). Type 1 and 2 diabetic mice both demonstrated fewer collateral arteries visible on angiography in the ischemic hind limb and no difference between diabetic groups was observed. Consequently, the angioscores of both diabetic groups were lower than that of the control group.

Effect of ischemia on angiogenesis. Capillary density was greater in the ischemic than the nonischemic hind limb in control mice (Fig 3, *A* and *B*). A similar pattern was evident in type 1 diabetic mice. In contrast, no difference between the ischemic and nonischemic limbs was present in type 2 diabetic mice; moreover, the level of capillary density in the nonischemic hind limb in this group was less than in the control group or type 1 diabetic mice.

Effect of ischemia on tissue integrity and muscle histology. The rate of incisional wound healing among the groups was similar, and all wounds healed completely by 5 to 7 days after surgery. No toe gangrene or digit autoamputation was evident. Histologic evidence of muscle necrosis was evident in the calf muscles from the ischemic hind limb in all groups, as shown by the presence of reduced myofiber number and size on hematoxylin and eosin staining (not shown). Oil Red O staining revealed extensive fat infiltration in the ischemic calf muscles from type 2 diabetic mice. Fat infiltration was never observed in the ischemic hind limb of control mice, and although present, was minimal in the ischemic calf muscles from type 1 diabetic mice (Fig 4). Neither fat infiltration nor muscle necrosis was observed in nonischemic muscles in any group.

Weights of the tibialis anterior (TA) and gastrocnemius (GC) muscles from the ischemic hind limb were different among the three study groups (grams): Control: TA, 1.04 ± 0.12; GC, 0.92 ± 0.15; type 1 diabetes: TA, 0.52 ± 0.03*; GC, 0.57 ± 0.09*; and type 2 diabetes: TA, 1.90 ± 0.45*†; GC, 1.15 ± 0.14† (*$P < .05$ type 1 or type 2 diabetic groups vs control group, †$P < .05$ type 2 diabetic group vs type 1 diabetic group). As determined from muscle histology, particularly the degree of fat, the greater muscle weight in type 2 diabetic mice was most likely due to fat infiltration rather than skeletal muscle recovery.

Effect of ischemia on muscle eNOS expression and nitrotyrosine accumulation. Expression of eNOS in the nonischemic thigh and gastrocnemius muscles from type 1 and type 2 diabetic mice was less than in control mice (Fig 5, *A* and *B*). After ischemia, eNOS expression significantly decreased in the hind limb muscles of all three groups, with one exception: expression of eNOS was not significantly different between ischemic and nonischemic gastrocnemius muscles from type 1 diabetic mice. Nitrotyrosine accumulation was significantly greater in the ischemic than nonischemic gastrocnemius and thigh muscles in all study groups (Fig 5, *C* and *D*). In the gastrocnemius muscle, nitrotyrosine accumulation was greater in both type 1 and type 2 diabetic mice than in control mice; moreover, nitrotyrosine expression in the ischemic gastrocnemius muscle was greater in type 2 than in type 1 diabetic mice. No differences between diabetic groups were noted in the thigh muscles.

Effect of ischemia on eNOS expression and nitrotyrosine accumulation in bone marrow-derived EPCs. Expression of eNOS in EPCs harvested from the bone marrow from the femur and tibia of the nonischemic limb was significantly less in type 1 or type 2 diabetic mice than in control mice (Fig 6, *A*). In control mice and type 1 diabetic mice, EPCs harvested from the femur and tibia of the ischemic hind limb demonstrated less eNOS expression than marrow-derived EPCs from the nonischemic hind limb. In contrast, eNOS expression in marrow-derived EPCs from the ischemic hind limb from type 2 diabetic mice was not different from the relatively low level present in the nonischemic limb in this group. Nitrotyrosine accumulation was greater in bone marrow-derived EPCs harvested from the ischemic hind limb in control mice and type 2 diabetic mice. No difference was noted in this variable in type 1 diabetic mice (Fig 6, *B*).

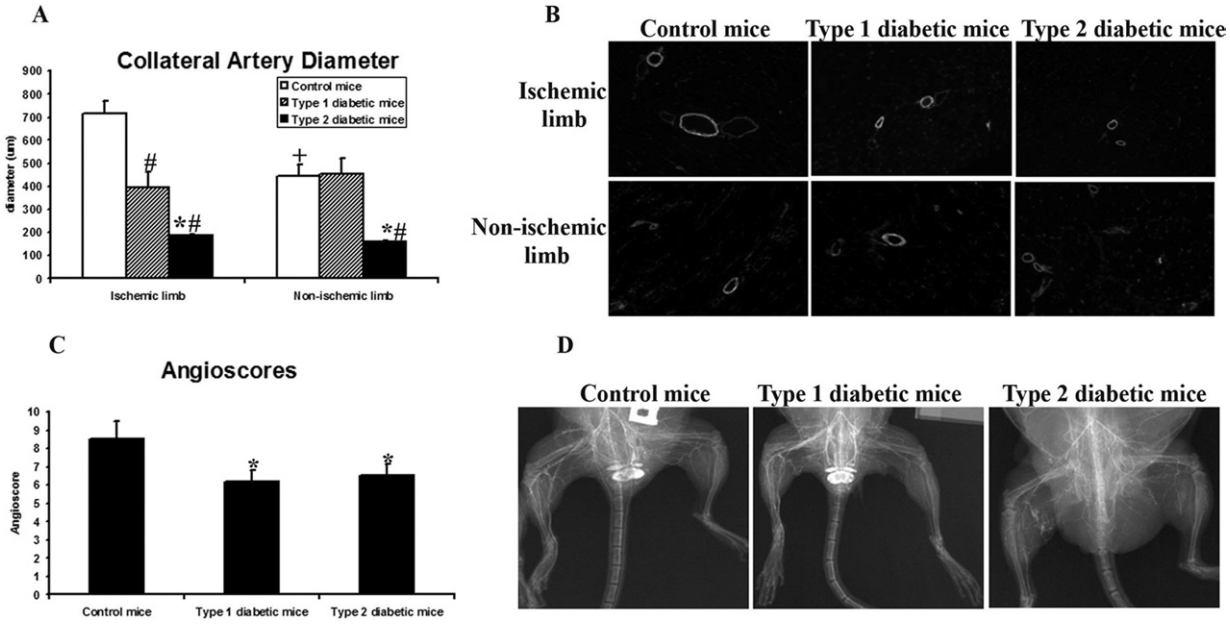

Fig 2. Effect of ischemia on collateral arteries was studied 28 days after the induction of ischemia (n = 5 animals in each group). **A,** Mean data for collateral artery diameter in thigh muscles are expressed in micrometers. The *error bars* indicate the standard deviation. $^{\#}P < .05$ for type 1 or type 2 diabetic mice vs control mice, $^{*}P < .05$ for type 2 vs type 1 diabetic mice, and $^{+}P < .05$ for ischemic vs nonischemic hind limb. **B,** In representative photomicrographs (original magnification ×200), CD31 is stained red, smooth muscle actin is stained green, and nuclei are stained blue. **C,** Data for angioscores represent the mean number of collateral arteries crossing fixed points on a grid superimposed on the radiograph (n = 5 animals in each group). The *error bars* indicate the standard deviation. $^{*}P < .05$ for type 1 or type 2 diabetic mice vs control mice. **D,** Representative radiographs are shown for control, type 1, and type 2 diabetic mice. Films were obtained 28 days after induction of hind limb ischemia. The radiographs depict the distal one third of the mouse.

Effect of ischemia on the concentration of EPCs in bone marrow and peripheral blood. The number of EPCs ($CD34^+$, $Flk\text{-}1^+$, $CD133^+$) in the bone marrow from the femur and tibia of the nonischemic hind limb was similar among the three study groups. All groups also demonstrated a greater number of bone marrow EPCs in the femur and tibia of the ischemic than nonischemic hind limb; however, the magnitude of this difference was significantly greater in control mice than in either diabetic group. Moreover, the percentage of EPCs in bone marrow from the ischemic hind limb was greater in type 2 than type 1 diabetic mice (Fig 7, *A*). The EPC concentration in peripheral blood was greater in control mice than in either diabetic group, and this value was significantly less in type 2 than in type 1 diabetic mice. The concentration of EPCs in the peripheral blood increased after induction of ischemia in control mice. In contrast, this level decreased in type 1 diabetic mice and remained unchanged in type 2 diabetic mice. As a consequence, the peripheral blood EPC concentration after induction of ischemia was significantly lower in both diabetic groups than in control mice (Fig 7, *B*).

Effect of ischemia on EPC function. VEGF-induced EPC migration was significantly greater in control mice than in type 1 or type 2 diabetic mice (Fig 8, *A*). EPCs from both type 1 and type 2 diabetic mice showed significantly impaired EPC incorporation into tubular structures than in control mice. However, the magnitude of this impairment was greater in EPCs from type 2 than from type 1 diabetic mice (Fig 8, *C*).

DISCUSSION

The present report offers the first evidence, to our knowledge, that type 1 and type 2 diabetic mice demonstrate different vascular responses to severe hind limb ischemia under identical experimental conditions. This difference was best evidenced by less effective hind limb blood recovery in type 2 than in type 1 diabetic mice; in addition, marked adipocyte infiltration was evident in the ischemic hind limb muscle of type 2, but not type 1 mice. We propose that differences in eNOS expression and oxidant stress within ischemic muscle and in EPCs, as well as differences in EPC function between type 1 and type 2 diabetic mice, partly explain the disparate vascular responses between groups.

Arteriogenesis is defined as the anatomic enlargement of collateral artery diameter in response to occlusion or obstruction of a major conduit artery.[21] The physiologic effect of this remodeling is increased collateral vascular conductance that results in the restoration of downstream blood flow.[21] This definition was fully satisfied in the

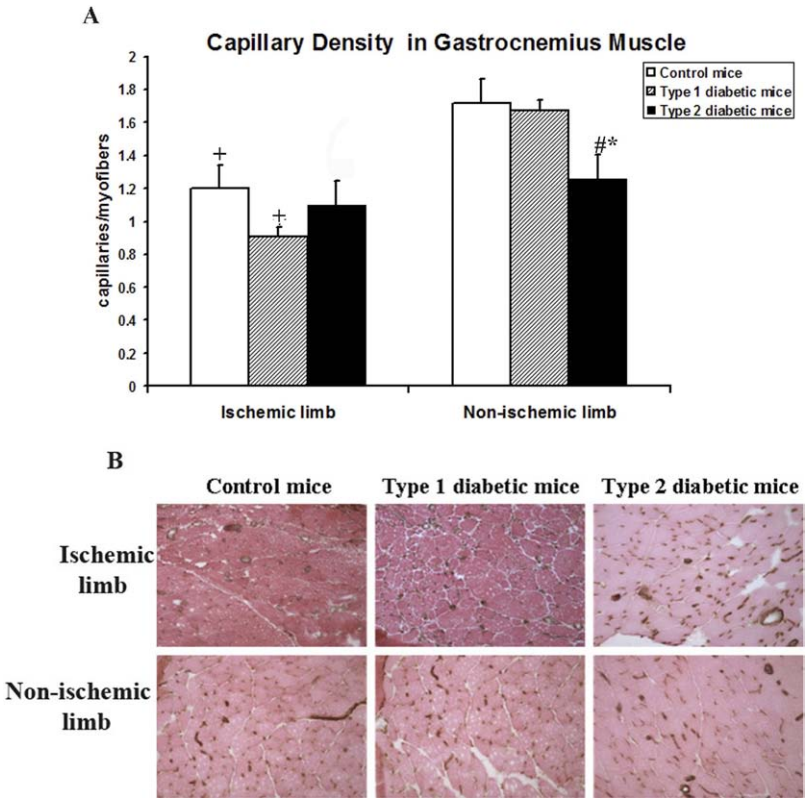

Fig 3. Effect of ischemia on capillary density was studied 28 days after the induction of ischemia ($n = 5$ animals in each group). **A,** Data for capillary density in gastrocnemius muscle are expressed as the mean number of $C31^+$ cells per myofiber, with *error bars* showing the standard deviation. $^{\#}P < .05$ for type 1 or type 2 diabetic mice vs control mice, $^{*}P < .05$ for type 2 vs type 1 diabetic mice, and $^{+}P < .05$ for ischemic vs nonischemic hind limb. **B,** In these representative photomicrographs (original magnification ×200), $CD31^+$ cells are stained brown.

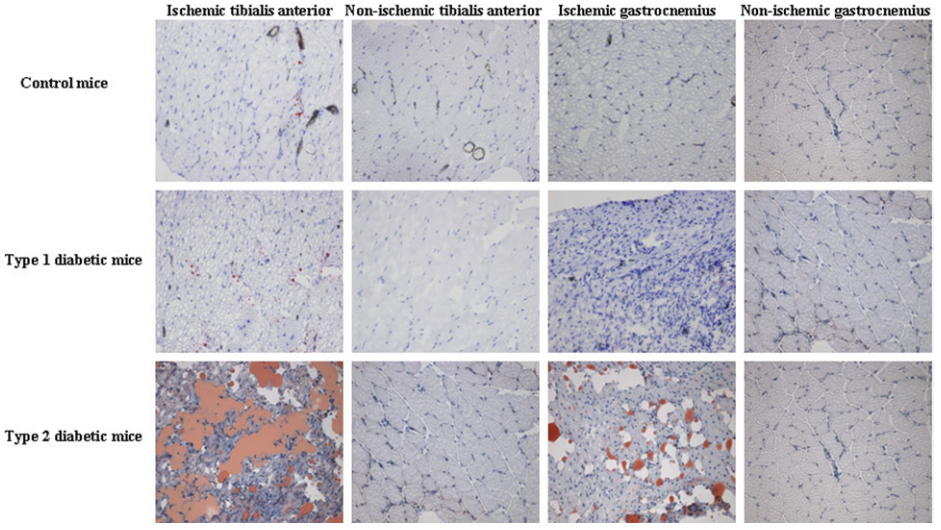

Fig 4. Photomicrographs (original magnification ×200) show representative Oil Red O staining of hind limb muscles harvested 28 days after induction of ischemia, in which fat is stained red; counterstained with hematoxylin. These photomicrographs are representative of observations made in 5 animals from each group.

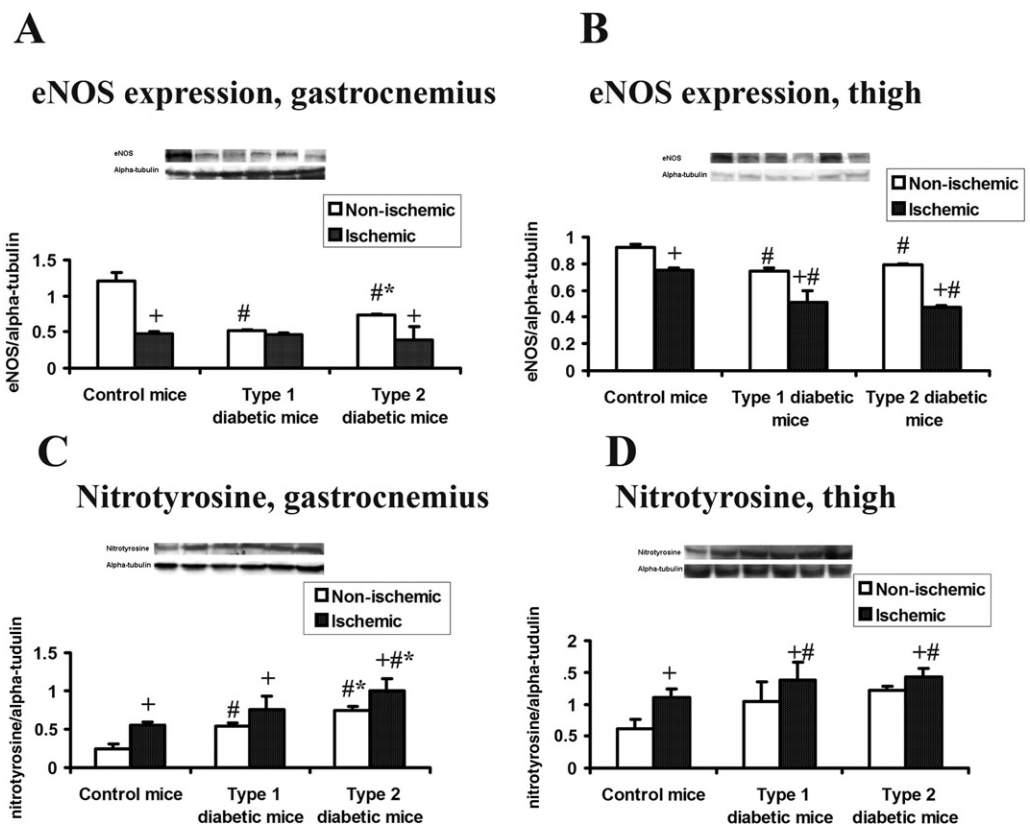

Fig 5. Endothelial nitric oxide synthase *(eNOS)* and nitrotyrosine expression was measured 28 days after induction of ischemia in hind limb muscle (n = 3 mice in each group). Mean data are presented with the standard deviation *(error bars)*. $^{\#}P < .05$ for type 1 or type 2 diabetic mice vs control mice, $^{*}P < .05$ for type 2 vs type 1 diabetic mice, and $^{+}P < .05$ for ischemic vs nonischemic hind limb. **Insets,** Representative Western blots; lane designations from left to right: control, nonischemic hind limb; control, ischemic hind limb; type 1 diabetes, ischemic hind limb; type 1 diabetes, nonischemic hind limb; type 2 diabetes, nonischemic hind limb; type 2 diabetes, ischemic hind limb. Expression of eNOS is shown in the **(A)** gastrocnemius and **(B)** thigh muscles. Nitrotyrosine expression is shown in the **(C)** gastrocnemius and **(D)** thigh muscles.

ischemic limb of control mice, indicating the presence of arteriogenesis in this group; thus, collateral diameter was 58% greater in the ischemic vs the nonischemic hind limb, whereas foot perfusion was restored to 65% of baseline.

The response was less clear in the diabetic groups. Collateral artery diameter was smaller in type 2 than in type 1 diabetic mice in both the ischemic and the nonischemic hind limb. The latter finding might indicate that the effect of type 2 diabetes on collateral artery dimension is long-standing, that is, that it might reflect an effect on collateral artery development, possibly a deficiency in the responsiveness to shear stress, a critical stimulus for collateral development.[21]

Anatomic evidence of arteriogenesis in response to ischemia was not present in type 1 or type 2 diabetic mice, because neither group demonstrated a difference in collateral diameter between the ischemic and nonischemic hind limbs, a finding consistent with the studies of Schiekofer et al[22] in *db/db* mice and Tamarat et al[5] in streptozotocin-treated mice. However, we found that postischemic recovery of hind limb perfusion was clearly greater in type 1 than in type 2 diabetic mice; that is, there was a disparity between anatomic (collateral diameter) and physiologic (hind limb perfusion) data. Reconciliation of this inequality cannot be achieved from existent findings. We speculate that a more concentrated study of collaterals, categorizing vessel dimension as a function of anatomic location between specific muscles or increasing the number of collaterals measured in each mouse, might generate anatomic evidence of arteriogenesis in type 1 diabetic mice.

Angiogenesis is defined as the de novo generation of capillaries in tissue downstream from the site of conduit vessel occlusion or obstruction.[21] The capillary/myofiber ratio was less in the ischemic than in the nonischemic gastrocnemius muscle from control mice and type 1 diabetic mice, indicating that postischemic angiogenesis did not occur in either group. Type 2 diabetic mice exhibited a ratio lower than type 1 or control mice in both ischemic and nonischemic gastrocnemius muscles, signifying a reducing capillary density in these mice. Interestingly, in contrast to

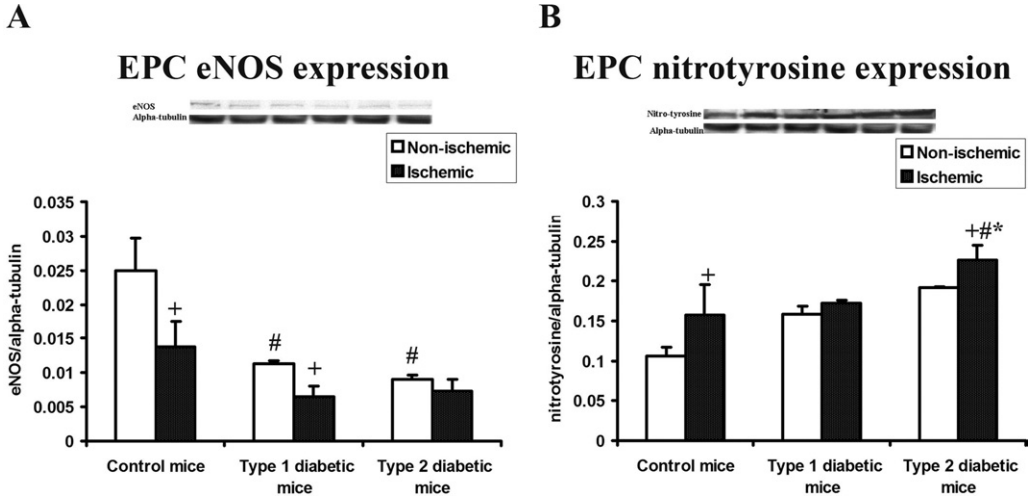

Fig 6. Expression of **(A)** endothelial nitric oxide synthase *(eNOS)* and **(B)** nitrotyrosine in bone marrow-derived epithelial progenitor cells (EPCs) was measured. EPCs were harvested from the bone marrow derived from the femur and tibia from ischemic and nonischemic hind limbs 7 days after induction of ischemia (n = 3 animals in each group). Mean data are presented with the standard deviation *(error bars)*. $^{\#}P < .05$ type 1 or type 2 diabetic mice vs control mice, $^{*}P < .05$ type 2 vs type 1 diabetic mice, and $^{+}P < .05$ ischemic vs nonischemic hind limb. **Insets,** Representative Western blots; lane designations from left to right: control, nonischemic hind limb; control, ischemic hind limb; type 1 diabetes, ischemic hind limb; type 1 diabetes, nonischemic hind limb; type 2 diabetes, nonischemic hind limb; type 2 diabetes, ischemic hind limb.

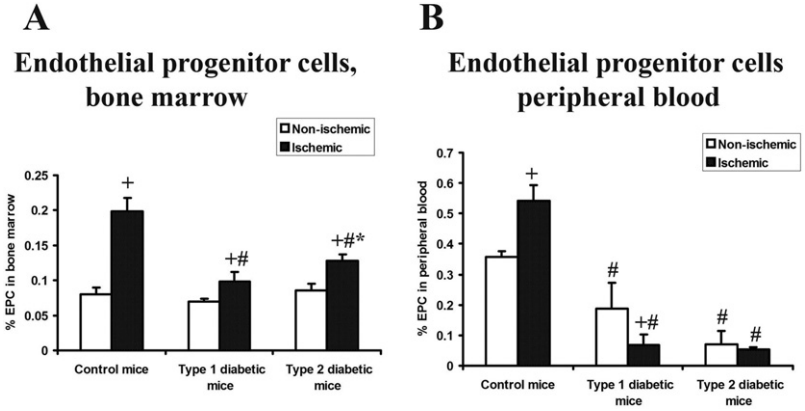

Fig 7. The percentage of endothelial progenitor cells *(EPCs)* was measured in bone marrow and peripheral blood samples harvested from the femur and tibia of the nonischemic or ischemic hind limbs 7 days after induction of ischemia (n = 6 animals per group). EPCs were characterized as $CD34^{+}$, $Flk\text{-}1^{+}$, $CD133,^{+}$ and cell quantitation was by fluorescence-activated cell-sorting analysis. Mean data are shown with the standard deviation (*error bars*). $^{\#}P < .05$ for type 1 or type 2 diabetic mice vs control mice, $^{*}P < .05$ for type 2 vs type 1 diabetic mice, and $^{+}P < .05$ for ischemic vs nonischemic hind limb.

type 1 diabetic or control mice, the capillary/myofiber ratio in type 2 mice was similar between the ischemic and nonischemic hind limbs. One interpretation of this finding is that the existent capillary density was protected in type 2 mice, an effect that might reflect the reduced postischemic perfusion recovery in type 2 mice.

Tissue oxygen delivery is directly proportional to flow rate, and one compensation for reduced oxygen delivery is increased capillary density, which yields an increased rate of diffusion of available capillary oxygen to cells.[23] Reduced tissue oxygen delivery also increases tissue hypoxia, the primary stimulus for angiogenesis.[24] Type 2 diabetic mice experienced the lowest postischemic flow rate, and we speculate that this circumstance may have provided a greater stimulus for angiogenesis, thus precluding the reduction in the capillary/myofiber ratio noted in the ischemic gastrocnemius in type 1 diabetic and control mice. It remains, however, that type 2 diabetic mice did not dem-

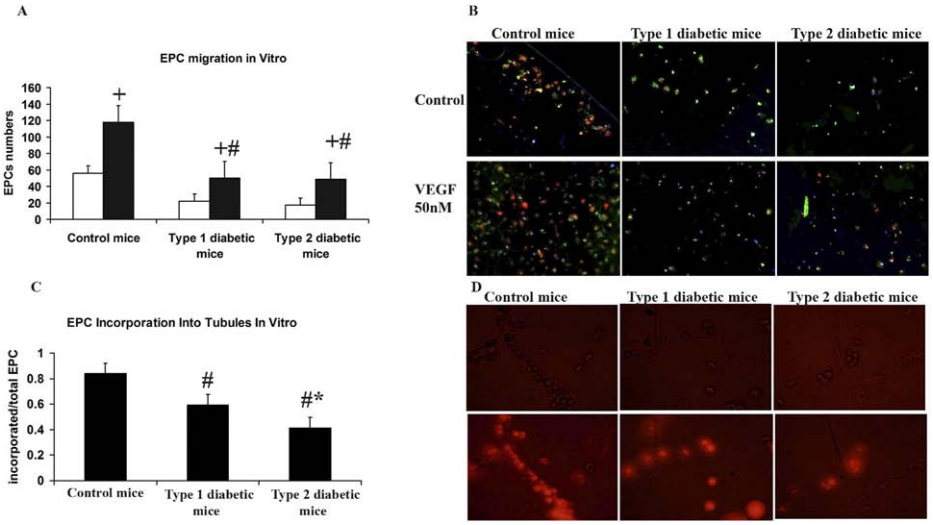

Fig 8. In vitro functional assays of endothelial progenitor cells *(EPCs)* harvested from the femur and tibia of ischemic and nonischemic hind limbs 7 days after the induction of ischemia and grown in culture for 3 days *($n = 6$* animals in each group). **A,** EPC migration assay. The *white bars* represent migration in response to medium alone (baseline), and the *black* bars represent migration in response to vascular endothelial growth factor *(VEGF)*. Mean data are presented with the standard deviation *(error bars)*. $^{+}P < .05$ for migration in response to VEGF vs migration in response to medium, $^{\#}P < .05$ for type 1 or type 2 diabetic mice vs control mice. **B,** Representative photomicrographs (original magnification ×200) of migration assay. UE lectin is stained green, Dil-Ac-LDL is stained red, and nuclei are stained blue. **C,** Incorporation of EPCs into tubular structures ($n = 6$ animals in each group). Mean data are presented with the standard deviation *(error bars)*. $^{\#}P < .05$ for type 1 or type 2 diabetic mice vs control mice. **D,** Representative photomicrographs (original magnification ×200) of tubular incorporation studies. The *upper panels* are bright field images. The *lower panels* are fluorescent images. EPCs are stained red.

onstrate an increased capillary/myofiber ratio in the ischemic vs nonischemic muscle, and in the strictest sense, we cannot conclude that postischemic angiogenesis occurred in this group.

EPCs are bone marrow-derived progenitor cells that are mobilized into the peripheral blood circulation in response to vascular injury and are essential participants in both arteriogenesis and angiogenesis within ischemic tissue.[7-9] Before induction of hind limb ischemia, the percentage of EPCs in peripheral blood was lower in type 1 and type 2 diabetic mice than in the control group, despite the presence of similar levels of these cells in bone marrow before induction of ischemia in all three groups. This finding is consistent with observations previously made in patients with type 1 diabetes, in which the percentage of circulating EPCs was noted to be depressed, and the severity of diabetic vasculopathy was inversely correlated with the presence of EPCs in peripheral blood.[25] The percentage of EPCs in peripheral blood in control mice increased 39% after induction of ischemia. This percentage decreased in type 1 and type 2 diabetic mice at this time, however, indicating a failure of bone marrow mobilization of EPCs in response to hind limb ischemia in both types of diabetic mice. Fadini et al[11] and Gallagher et al[10] reported a similar finding in streptozotocin-induced type 1 diabetes in rats and mice, respectively. To our knowledge, however, the present report provides the first evidence of impaired EPC mobilization in type 2 diabetic mice.

Impairment of EPC in vitro function has been reported in patients with type 1 and type 2 diabetes.[12,13] The present findings indicate impaired VEGF-induced migration in marrow-derived EPCs from type 1 and 2 diabetic mice, as well as impaired incorporation of EPCs into tubular structures that was less effective in type 2 than in type 1 diabetic mice.

EPCs participate in both arteriogenesis and angiogenesis.[7] Impairment of EPC mobilization and function was evident in both type 1 and type 2 diabetic mice, and these effects may explain, in part, the lack of a postischemic increase in collateral artery diameter (arteriogenesis) and capillary/myofiber ratio (angiogenesis) in these study groups. Huang et al[26] reported compromised arteriogenesis and angiogenesis in KKAy mice, a genetic model of type 2 diabetes. This group also noted significant improvement in the postischemic vascular response, in association with normalization of VEGF levels, and increased activation of eNOS and Akt after treatment with pioglitazone, a proliferator-activated receptor-γ agonist. In this context, the reduced EPC eNOS expression noted in both type 1 and type 2 diabetic mice may possibly have contributed to the deficiency in arteriogenesis and angiogenesis in these mice and that treatment with a thiazolidinedione, such as pioglitazone, might restore these postischemic responses.

An original and important observation made in this study was that extensive fat infiltration occurred in the ischemic calf muscles of type 2 diabetic mice. The *db/db*

mice are obese,[18] and it might thus be argued that the observed fat infiltration was a reflection of this obesity; however, this fat infiltration only occurred in the ischemic hind limb. We speculate that this change may reflect adipogenic differentiation of mesenchymal stem cells (MSCs). MSCs are bone marrow-derived progenitor cells that, like EPC, home to the site of vascular injury where they participate in arteriogenesis and angiogenesis.[27,28] MSCs are pluripotent cells that can differentiate into several cell types, including chondrocytes, adipocytes, and endothelial cells.[29] We propose that MSCs that homed to the ischemic hind limb in type 2 mice were stimulated to an adipocytic lineage, a fate that would effectively nullify their involvement in vascular repair or adaptation.

Multiple mouse models of type 1 and type 2 diabetes have been described. We selected the streptozotocin-induced hypoinsulinemic mouse as a model of type 1 diabetes because it reproducibly duplicates the rapid development of β-cell insulinitis and subsequent hypoinsulinemia characteristic of human type 1 diabetes.[18] An alternative model is the nonobese diabetic mouse, a genetic model of type 1 diabetes.[30,31] This mouse, however, does not manifest full expression of the diabetic phenotype until 4 to 5 months of age, and this expression is not robust in male mice. These conditions were deemed unacceptable because we wished to generate hind limb ischemia at 2 to 3 months of age in male mice to facilitate the comparison of our data with published reports.

We selected the *Lepr$^{db/db}$* mouse as a model for type 2 diabetes because it recapitulates the insulin resistance, moderate hyperglycemia, and atherogenic dyslipidemia characteristic human type 2 diabetes. A potential disadvantage of the *db/db* mouse is that leptin may directly affect the proinflammatory immune responses[32] and affect T-cell function,[33] phenomena that may be relevant in angiogenesis.[34] We recognize that animal models that faithfully duplicate human type 1 and type 2 diabetes do not exist. We contend, however, that this caveat does not nullify the importance of the present findings, particularly in light of the frequency with which others have used these diabetic mouse models.

An important methodologic caveat is the means used to generate hind limb ischemia. We used extirpation of the femoral artery, a commonly used experimental model but one that generates acute severe limb ischemia and inflammation.[4] This circumstance does not recreate the progressive narrowing of the iliac, femoral, or popliteal arteries that characterizes peripheral arterial disease, and a comparison between acute vs chronic occlusion of the femoral artery in rats indicates that muscle hypoxia and necrosis are greater and angiogenesis less effective after acute occlusion.[20] Unfortunately, the ameroid constrictor used to generate chronic hind limb ischemia in that study is too large for use in mice. It is possible that the lack of postischemic collateral artery enlargement (arteriogenesis) noted in diabetic mice may reflect the abrupt generation of ischemia, insofar as collateral development appears to occur in diabetic individuals with peripheral arterial disease.

A second caveat to the study design was the use of the contralateral, nonischemic hind limb as an internal control for each mouse. It is possible that incision of a hind limb and subsequent femoral artery extirpation therein generates a systemic response that affects the contralateral, nonoperated on hind limb. Indeed, this effect might explain why less muscle eNOS expression was found in the contralateral, nonischemic hind limb in type 1 and type 2 diabetic mice vs the control group.

CONCLUSIONS

The vascular response to hind limb ischemia in diabetic mice is contingent on the type of diabetes present. Type 2 diabetic mice demonstrated significantly less restoration of hind limb perfusion 28 days after induction of hind limb ischemia. Both type 1 and type 2 diabetic mice failed to mobilize bone marrow-derived EPCs to the peripheral circulation in response to hind limb ischemia, whereas type 2 diabetic mice displayed greater reduction of EPC function in vitro (incorporation into tubules) than type 1 diabetic mice.

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AUTHOR CONTRIBUTIONS

Conception and design: JY, LM
Analysis and interpretation: JY, LM
Data collection: JY, GT, BP, YY
Writing the article: JY, PN
Critical revision of the article: PN, LM
Final approval of the article: LM
Statistical analysis: JY
Obtained funding: LM
Overall responsibility: LM

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