Type-2 diabetic Lepr\textsuperscript{db/db} mice show a defective microvascular phenotype under basal conditions and an impaired response to angiogenesis gene therapy in the setting of limb ischemia

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1. ABSTRACT

Diabetes mellitus is associated with macro- and micro-angiopathy, leading to increased risk of peripheral ischemia. In the present study, we have characterized the microvascular phenotype at the level of limb muscles and the spontaneous angiogenesis response to surgically-induced unilateral limb ischemia in a murine model of type-2 diabetes, the obese C57BL/KsOlaHsd-Lepr\textsuperscript{db/db} mice (Lepr\textsuperscript{db/db}), and in non-diabetic heterozygous Lepr\textsuperscript{db/+}. Wild type C57BL mice (WT) were used as controls. The basal microvascular phenotype was determined in mice aged 3 or 5 months, while the response to limb ischemia was studied only in 5-month old mice. Moreover, in 5-month old ischemic Lepr\textsuperscript{db/db} and Lepr\textsuperscript{db/+}, we have tested the therapeutic potential of local angiogenesis gene therapy with human tissue kallikrein (hTK) or constitutively-activated Akt kinase (Myr-Akt). We found that in the muscles of 3- or 5-month old Lepr\textsuperscript{db/db}, apoptosis of endothelial cells was enhanced and the densities of capillary and arteriole were reduced. Arterioles of Lepr\textsuperscript{db/db} showed hypertrophic remodelling and, occasionally, lumen occlusion. Following ischemia, Lepr\textsuperscript{db/db} showed a defective reparative angiogenesis in ischemic muscle, delayed blood flow recovery, and worsened clinical outcome as compared with controls. Five-month old Lepr\textsuperscript{db/+} displayed an increase in endothelial cell apoptosis under basal conditions, while capillary and arteriole densities were normal. Lepr\textsuperscript{db/+} mounted a proper reparative angiogenesis response to limb ischemia and regained blood flow to the ischemic limb, regularly. Local gene therapy with hTK or Myr-Akt induced angiogenesis in ischemic muscles of Lepr\textsuperscript{db/+} and Lepr\textsuperscript{db/db}. However, in the Lepr\textsuperscript{db/db} neither gene therapy approach improved the blood flow recovery and the clinical outcome from ischemia. In contrast, either hTK or Myr-Akt gene transfer improved the post-ischemic recovery of Lepr\textsuperscript{db/+}. Type-2 diabetes has a negative impact on the basal microvascular phenotype and severely impairs post-ischemic recovery of limb muscles. Gene therapy-induced stimulation of neovascularization might not suffice as a sole therapeutic strategy to combat type-2 diabetes-related vascular complications. In type-2 diabetic patients, therapeutic angiogenesis may need to be further optimized before being recommended for clinical applications.
2. INTRODUCTION

Type-2 diabetes accounts for 90-95% of all diabetes. The disorder affects around 6% of the adult population in industrialized countries and its worldwide prevalence is expected to increase at the rate of 6% per year, reaching a total of 200-300 million cases by 2010 (1,2). Type-2 diabetes is associated with endothelial dysfunction, atherosclerotic macrovascular disease, and microangiopathy. As a consequence, patients show a much higher risk of limb amputation, myocardial infarction, and stroke. Cardiovascular complications are eventually responsible for 80% of casualties and more than 75% of all hospitalizations related to diabetes (3).

Diabetes impairs the reparative neovascularization response to arterial occlusion, thus accounting for the worse clinical outcome of diabetic patients following an ischemic accident (4). Supply-side approaches with angiogenic substances or endothelial cell (EC) precursors have been successfully applied for rescuing ischemia in type-1 diabetic rodents (5-7). In addition, prophylactic delivery of the human tissue kallikrein gene (hTK) reportedly halts the progression of microvascular rarefaction in adductor muscles of streptozotocin-induced type-1 diabetic mice (8) and ensures an improved haemodynamic recovery in case of supervening arterial occlusion (9). However, the successes achieved by therapeutic angiogenesis in mice, dogs and pigs have not been replicated by clinical trials conducted to date (10). One major reason for these discrepancies consists of the fact that models relying on young, mildly compromised animals might be inadequate to reproduce the real situation of patients with advanced occlusive arteriolar disease and associated risk factors. Appropriate models should therefore be considered in order to obtain unequivocal proof of efficacy before clinical testing.

The present study was conducted in the type-2 diabetic and obese C57BL/KsOlaHsd-Leprdb/db mice (Leprdb/db). We also investigated the angiogenesis phenotype of heterozygous mice (Leprdb/+). Leprdb/db carry a genetic mutation of the leptin receptor, which inactivates the receptor signalling. Leprdb/db develop severe diabetes associated with hyperphagia, obesity, and hyperinsulinemia (11), while Leprdb/+ have a normal phenotype. Although a genetic mutation similar to that of Leprdb/db does not exist in humans, mutant mice share many characteristics of type-2 diabetic patients, including obesity, uniquely impaired healing response to tissue injury, and insensitivity to increased blood leptin levels (12-15). Leprdb/db were already superficially investigated for their capacity to mount a reparative response to peripheral ischemia (16,17). In the present paper, we have characterized the basal microvascular phenotype in limb muscles of 3- and 5-month old Leprdb/db and Leprdb/+ and the angiogenesis and apoptosis responses to peripheral ischemia in 5-month old Leprdb/db and Leprdb+/. In previous work, we showed that hTK potently stimulates angiogenesis through kinin-mediated activation of the PI3K-Akt-NO pathway (18,19). Akt, a kinase that lies downstream of various proangiogenic and anti-apoptotic agents (20-26), reportedly promotes reparative angiogenesis in animal models (27). Based on this background, we have challenged the therapeutic potential of angiogenesis gene therapy with hTK or constitutively activated Akt (Myr-Akt) in ischemic Leprdb/db and Leprdb+/

3. MATERIALS AND METHODS

3.1. Animal Model

Procedures complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md, 1996) and were approved by the INBB Ethical Committee and by the Italian Minister of Health. All the in vivo work was performed at the INBB. Homozygous male C57BL/KsOlaHsd-Leprdb/db mice (Leprdb/db, Harlan, Milan, Italy) and non-diabetic Leprdb/+ were studied at 3 and 5 months of age. Age-matched wild type C57BL (WT) mice served as controls. Body weight (BW), fasting plasma glucose levels, and glycosuria were monitored throughout the study.

3.2. Assessment of the Time Course of Microangiopathy

Adductor capillary density and arteriole profile and density were determined, as previously described (8,9,16), in mice aged 3 or 5 months (n=8 mice per group). At the same time points, apoptosis of EC and myofiber was evaluated by a terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay on paraffin-embedded muscular sections, as described (8,18).

3.3. Gene Constructs

Adenovirus (Ad) carrying hTK (Ad.CMV-hTK) (28) or the constitutively-active mutant Myr-Akt (Ad.Myr-Akt-B) (29) was used. An adenovirus harbouring the luciferase gene (Ad.CMV-Luc) served as a control.

3.4. Ischemia Model and Gene Delivery

Left limb ischemia was induced in anesthetized (2,2,2-tribromoethanol –avertin, 880 mmol/kg body wt IP, Sigma-Aldrich, Italy) Leprdb/db, Leprdb/+ and WT mice. To this aim, the left femoral artery was dissected free from the femoral vein and nerve and it was ligated with 6.0 silk just below the inguinal ligament. The arterial segment (0.5 mm in length) below the ligation was electrocoagulated. This operative procedure is suited to limit spontaneous autoregulation rate and avoid sufficiency to the animals (9). In another experiment, 5-month old Leprdb/db and Leprdb/+ were submitted to ischemia and injected with one of the adenoviruses (1x10⁸ plaque-forming units [p.f.u.] in 10 µL) or saline into 3 sites of the left adductor alongside the femoral artery. This procedure enables the Ad vector incorporation across all the adductor (18). The capacity of Ad.hTK and Ad.Myr-Akt to infect the ischemic adductor muscle of Leprdb/db was verified. Realtime RT-PCR was performed on extracts from Leprdb/db ischemic muscles injected with Ad.hTK or Ad.Luc to evaluate the human tissue kallikrein transgene expression using specific primer pairs (hTK sense: 5'-TGCACAGAGCCTGCTGATACC-3', hTK antisense: 5'-TCACCCACACAGGTGTCTTT-3') and the LightCyclerTM technology (Roche Diagnostics, UK). The amount of hTK mRNA was normalized to the amount...
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Table 1. Body Weight and Hemodynamic Parameters

<table>
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<tr>
<th></th>
<th>WT</th>
<th>Lepr&lt;sup&gt;db/db&lt;/sup&gt;</th>
<th>Lepr&lt;sup&gt;ad/db&lt;/sup&gt;</th>
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<tr>
<td>BW (g), 3 months</td>
<td>23±1</td>
<td>28±2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>52±2&lt;sup&gt;**&lt;/sup&gt;±0&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>5 months,</td>
<td>29±2</td>
<td>30±2</td>
<td>55±2&lt;sup&gt;**&lt;/sup&gt;±0&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>BP (mmHg), 3 months</td>
<td>109±4</td>
<td>106±6</td>
<td>105±11</td>
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<tr>
<td>5 months</td>
<td>122±4</td>
<td>128±12</td>
<td>126±12</td>
</tr>
<tr>
<td>HR (b/min), 3 months</td>
<td>698±14</td>
<td>528±5&lt;sup&gt;†&lt;/sup&gt;</td>
<td>529±50&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>3 months</td>
<td>675±14</td>
<td>515±8&lt;sup&gt;†&lt;/sup&gt;</td>
<td>553±55&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>Glucosuria, 3 months</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
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<tr>
<td>5 months</td>
<td>Absent</td>
<td>Absent</td>
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Values are means±SEM. Each group consisted of 8 mice. BW, Body Weight; BP, Tail-Cuff Blood Pressure; HR, Heart Rate. *<i>P</i>&lt;0.05 and **<i>P</i>&lt;0.01 vs. WT; †<i>P</i>&lt;0.01 vs. Lepr<sup>ad/db</sup>.

of 18S rRNA (sense: 5'-TAGAGGGACAAGTGGCGTTC-3'; antisense: 5'TGTACAAAGGGCAGGG ACTT-3') in the same sample. Western blot for phosphorylated AktS473 (Cell Signalling, 1:1000) and total Akt (Cell Signalling, 1:1000) was performed on proteins extracted from Ad.Myr.Akt- and Ad.Luc-injected muscles. The housekeeping protein GAPDH (RDI, 1:1000) was used as a loading control.

3.6. Haemodynamic Measurements and Assessment of the Clinical Outcome

Systolic blood pressure (SBP) and heart rate (HR) were measured in unanesthetized mice by tail-cuff plethysmography (Visitech Systems) (30). Blood flow (BF) to the hindlimbs was measured by laser Doppler flowmetry (Lisca Inc., Sweden). BF measurements were performed on anesthetized mice prior to surgery and weekly thereafter. To determine the rate of blood flow (BF) recovery to the ischemic foot, the ischemic to non-ischemic foot BF ratio was calculated. The rate of foot autonecrosis was evaluated at 14 days from ischemia.

3.7. Histological Analyses of Ischemic Muscles

At 2 weeks post-ischemia, anesthetized mice (n=8 per group) were perfusion-fixed. Adductor muscles were harvested and processed for analysis of capillary and arteriole density and apoptosis.

3.8. Statistical Analysis

All results are expressed as mean±SEM. Statistical analyses were performed by the Sigmastat program. One-way ANOVA for comparison among groups were performed. In case ANOVA indicated significant difference among groups, Tukey post test was used to check for difference between groups. A <i>P</i> value &lt;0.05 was interpreted to denote statistical significance.

4. RESULTS

4.1. Cardiovascular Phenotype of Lepr<sup>db/db</sup>

As shown in Table 1, the BW of 3- or 5-month-old Lepr<sup>db/db</sup> was twice that of WT (P&lt;0.01), while Lepr<sup>ad/db</sup> displayed a modest overweight at 3 months only (P&lt;0.05, vs WT). Systemic blood pressure was similar among groups. The heart rate of Lepr<sup>db/db</sup> and Lepr<sup>ad/db</sup> was lower than that of WT (P&lt;0.05 for both comparisons). Overt glycosuria was constantly detected in Lepr<sup>db/db</sup>, but absent in Lepr<sup>ad/db</sup> and WT.

4.2. Microangiopathy of Lepr<sup>db/db</sup>

In limb muscles of 3- and 5-month old Lepr<sup>db/db</sup>, capillary density was lower than in WT (P&lt;0.01 for both comparisons) (Figure 1A). Similarly reduced (P&lt;0.01) was the capillary to myofiber ratio (Figure 1B). Lepr<sup>ad/db</sup> showed normal adductor muscle capillarity (P=N.S. vs WT for comparisons at 3 and 5 months). As shown in Figure 1C, arteriole density was reduced in Lepr<sup>db/db</sup> (9.1±1.1 vs. 23.0±2.1 art/mm<sup>2</sup> in WT at 3 months; 8.1±1.6 vs. 22.6±1.4 art/mm<sup>2</sup> in WT at 5 months, P&lt;0.01 for both comparisons). In Lepr<sup>db/db</sup>, microvessel rarefaction was associated with increased EC apoptosis (Figure 1D). In Lepr<sup>ad/db</sup>, apoptosis was also activated at myofiber level (26±4 vs 6±1 TUNEL-positive myofibers/mm<sup>2</sup> in WT, P&lt;0.01, Figure 1D). In Lepr<sup>ad/db</sup>-treated mice, the number of apoptotic ECs did not differ from the figure seen in WT at 3 months. However, EC apoptosis was increased at 5 months (P&lt;0.01 vs WT, Figure 1D). Capillary and arteriole density was normal in the muscles of 3- and 5-month old Lepr<sup>ad/db</sup>.

4.3. Impaired Reparative Angiogenesis in Lepr<sup>db/db</sup>

We then examined the ability of 5-month old Lepr<sup>db/db</sup> and Lepr<sup>ad/db</sup> to mount a spontaneous reparative angiogenesis response to interruption of femoral blood flow. Ischemic WT were used for reference. As shown in Figure 2, in WT mice, induction of ischemia resulted in a 49% increase of adductor capillary density (1008±27 vs 680±22 cap/mm<sup>2</sup> in contralateral muscle, P&lt;0.01). A similar increment was observed in Lepr<sup>ad/db</sup> (1036±54 vs 752±49 cap/mm<sup>2</sup> in contralateral, P&lt;0.01). In contrast, Lepr<sup>db/db</sup> failed to mount reparative capillarization (62.1±14 vs. 570±19 cap/mm<sup>2</sup> in contralateral muscle, P=N.S.). This deficit was also evident when considering the capillary to myofiber ratio of Lepr<sup>db/db</sup> ischemic muscles (0.88±0.03 vs 1.58±0.08 in Lepr<sup>db/db</sup> and 1.66±0.07 in WT, P&lt;0.01 for both comparisons).

In ischemic muscles of Lepr<sup>db/db</sup>, arteriole number was less (10.5±1.8 art/mm<sup>2</sup>) than in WT (24.6±2.9 art/mm<sup>2</sup>, P&lt;0.05) or Lepr<sup>ad/db</sup> (28.4±2.4 art/mm<sup>2</sup>, P&lt;0.05). Arteriole density of Lepr<sup>ad/db</sup> was normal (P=N.S vs WT). As shown in Figure 3, EC apoptosis was higher in the ischemic adductors of Lepr<sup>db/db</sup> (55±12 TUNEL-positive EC/mm<sup>2</sup>) than in WT (8±1 TUNEL-positive EC/mm<sup>2</sup>, P&lt;0.01). EC apoptosis was also elevated in Lepr<sup>ad/db</sup> (28±10 TUNEL-positive EC/mm<sup>2</sup>, P&lt;0.05 vs WT). Myocyte apoptosis was increased in the ischemic muscles of Lepr<sup>db/db</sup> only (27±10 vs 6±1 TUNEL-positive myocytes/mm<sup>2</sup> in WT, P&lt;0.05).

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Figure 1. Time course of the changes in capillary (cap) density (A), capillary to myofiber ratio (B), arteriole (art) density (C), and endothelial cell apoptosis (D) in adductor muscles from Lepr<sup>db/db</sup> (db/db, grey columns) and Lepr<sup>db/+</sup> (db/+, black columns). Values in age-matched wild-type mice (WT, white columns) are shown for reference. Values are mean±SEM and number within each column represents sample size. **P<0.01 vs WT, §P<0.05 vs Lepr<sup>db/+</sup>.

Figure 2. Reparative angiogenesis is impaired in ischemic muscles of Lepr<sup>db/db</sup>. Capillary density of ischemic (I) and contralateral (C) adductors was analyzed at 2 weeks from ischemia induction. Values are mean±SEM and number within each column represents sample size. ##P<0.01 vs C; **P<0.01 vs WT; §§P<0.01 vs Lepr<sup>db/+</sup>

As shown in Figure 4, arterioles in ischemic muscles of Lepr<sup>db/db</sup> showed hypertrophic remodeling (Panel B) and, occasionally, lumen occlusion (Panel C). The peri-adventitial tissue was infiltrated with mononuclear cells. These alterations were not observed in the ischemic muscles of WT mice (Panel A).

4.4. Impaired Clinical Outcome and Delayed Haemodynamic Recovery in Lepr<sup>db/db</sup>

As shown in Figure 5A, BF recovery was significantly delayed in Lepr<sup>db/db</sup> (P<0.05 vs. WT). The trend observed in Lepr<sup>db/+</sup> denoted a mild impairment, yet it did not differ significantly from WT (P=0.20 at 7 days and P=0.24 at 14 days). The clinical outcome of Lepr<sup>db/db</sup> was consistent with haemodynamic data, showing impaired cicatrisation of surgical wounds and occurrence of foot necrosis in 62% of the mice. In contrast, limb salvage was constantly observed in the other 2 groups. Representative images of Lepr<sup>db/db</sup> (Panel B) and WT (Panel A) limbs at 14 days from ischaemia induction are shown in Figure 5B.

4.5. Down-regulation of VEGF-A and eNOS in Ischemic Limb Muscles of Lepr<sup>db/db</sup>

As shown in Figure 6, the VEGF-A mRNA level was 1.9-fold lower in ischemic muscles of Lepr<sup>db/db</sup> compared with WT (P<0.05). VEGF-A expression was
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Figure 3. Endothelial cell (EC) apoptosis (left side) is increased in the ischemic adductor muscles of Lepr\textsuperscript{db/db} and Lepr\textsuperscript{db/+}. Myocyte apoptosis (right side) is increased in Lepr\textsuperscript{db/+}, only. Apoptosis was determined by in situ TUNEL staining of ischemic muscles harvested at 2 weeks post-ischemia. Values are mean±SEM and number within each column represents sample size. **P<0.01 and *P<0.05 vs WT.

Figure 4. Representative HE-stained sections (captured at 400 X) from ischemic adductors of WT (A) and Lepr\textsuperscript{db/db} (B, C). Arterioles of diabetic mice are characterized by wall thickening (B) and lumen occlusion (B), and they are surrounded by monocytes infiltrating the interstitial space (A, B).

4.6. Effects of Angiogenesis Gene Therapy in Lepr\textsuperscript{db/db} and Lepr\textsuperscript{db/+}

As shown in Figure 7A, hTK mRNA was present in Ad.hTK-injected Lepr\textsuperscript{db/db} muscles, whereas no hTK gene product was present in samples obtained from Ad.Luc-given adductors. As shown in Figure 7B, Ad.Myr-Akt-injected muscles showed increased content of both phosphorylated AktS473 (phosphorylated AktS473 to GAPDH densitometry ratio: 1.42±0.14 in Ad.Myr.Akt vs 0.8±0.20 in Ad. Null, P<0.05) and total Akt (total Akt to GAPDH densitometry ratio: 1.05±0.13 in Ad.Myr.Akt vs 0.57±0.02 in Ad. Null, P<0.01).

As shown in Figure 8, both Ad.hTK and Ad.Myr-Akt promoted angiogenesis in the ischemic limb muscles of Lepr\textsuperscript{db/+} (Panel A) and Lepr\textsuperscript{db/db} (Panel B). Moreover, both Ad.hTK and Ad.Myr-Akt increased arteriole density in ischemic muscles of Lepr\textsuperscript{db/db} (Ad.hTK: 37±6 art/mm\textsuperscript{2}; Ad.Myr.Akt: 21±3 art/mm\textsuperscript{2}, P<0.05 for both comparisons vs Ad.Luc: 12±7 art/mm\textsuperscript{2}) and Lepr\textsuperscript{db/+} (Ad.hTK: 32±9 art/mm\textsuperscript{2}; Ad.Myr.Akt: 22±6 art/mm\textsuperscript{2}, P<0.05 for both comparisons vs Ad.Luc: 10±6 art/mm\textsuperscript{2}). However, analysis of arteriole profile in hTK- or Myr.Akt-transduced diabetic muscles revealed that gene therapy was ineffective in improving structural alterations, namely, wall thickening and arterial occlusion that represent typical features of this diabetic model (data not shown).

In the Lepr\textsuperscript{db/+}, hTK- or Akt-induced neovascularization translated into improved recovery of BF to the ischemic muscles (see Figure 9A). In strict contrast, in Lepr\textsuperscript{db/db}, angiogenesis gene therapy with either hTK or Myr.Akt completely failed to ameliorate BF recovery (see Figure 9B). Neither of the clinical outcome was ameliorated by gene therapy (data not shown).

5. DISCUSSION

In this study, we have shown that type-2 diabetic and obese Lepr\textsuperscript{db/+} are affected by limb muscle microangiopathy, consisting of rarefaction of capillaries and arterioles which is likely attributable to apoptotic loss of vascular cells. These animals are also characterized by impaired reparative neovascularization and delayed blood flow recovery following experimentally-induced limb ischemia. Heterozygous Lepr\textsuperscript{db/+} showed a milder phenotype, with EC apoptosis being activated at later stages.

The capillary rarefaction that we have observed in Lepr\textsuperscript{db/+} might significantly contribute to alter the path length for oxygen transport to myocytes, thus, ultimately leading to ischemia and additional activation of cell-death mechanisms. Accordingly, myofiber apoptosis was reduced by 1.4-fold in Lepr\textsuperscript{db/+}, but this change did not reach statistical significance (P=N.S. vs WT). ENOS was up-regulated by ischemia in WT (3.9-fold increase vs contralateral, P<0.01), and, to a lesser extent, in Lepr\textsuperscript{db/+} (2.0-fold increase vs contralateral, P=0.05). In contrast, the modulation of eNOS by ischemia was abrogated in Lepr\textsuperscript{db/db}.

In the Lepr\textsuperscript{db/+}, hTK- or Akt-induced neovascularization translated into improved recovery of BF to the ischemic muscles (see Figure 9A). In strict contrast, in Lepr\textsuperscript{db/db}, angiogenesis gene therapy with either hTK or Myr.Akt completely failed to ameliorate BF recovery (see Figure 9B). Neither of the clinical outcome was ameliorated by gene therapy (data not shown).
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Figure 5. A: Blood flow (BF) recovery following induction of unilateral limb ischemia in Lepr<sup>db/db</sup> (open squares), Lepr<sup>db/+</sup> (open triangles), and WT (open circles). The recovery of superficial limb blood, measured by Laser Doppler Flowmetry, was delayed in Lepr<sup>db/db</sup>. Values are mean±SEM. **P<0.01 and *P<0.05 vs WT. B: Representative images of post-ischemic limbs in WT (A, left) and Lepr<sup>db/db</sup> (B, right). Pictures were taken at 2 weeks from ischemia induction. Lepr<sup>db/db</sup> showed delayed healing of surgical wounds and limb necrosis was a frequent clinical feature in these diabetic mice.

Figure 6. VEGF-A (A) and eNOS (B) mRNA levels are reduced in ischemic muscles of Lepr<sup>db/db</sup>. Muscles were harvested 3 days after ischemia induction and gene expression was determined by quantitative RT-PCR. Values were then normalized by GAPDH. Values are mean±SEM and number within each column represents sample size. mP<0.01 and *P<0.05 vs C; *P<0.05 and **P<0.01 vs WT; #P<0.01 vs Lepr<sup>db/+</sup>.

Another common clinical feature of diabetic patients is the failure to mount a proper collateralization in response to arterial occlusion, thus resulting in delayed tissue healing and recurrent ischemia (4). Similarly, reparative angiogenesis response was severely impaired in Lepr<sup>db/db</sup> and associated with arteriole remodeling and thrombosis. The latter phenomena are likely to account for inadequate reperfusion and excess of foot necrosis that we have observed in this strain. Impaired post-ischemic recovery of Lepr<sup>db/db</sup> was previously reported by us and, later, by Schiekofer et al (16, 17). These preliminary observations have been significantly expanded by the present study, showing arteriole remodeling, intra-luminal thrombosis, and uniquely activated apoptosis in vascular and muscular cells of Lepr<sup>db/db</sup> limb muscles following arterial occlusion. We have also evaluated the effect of heterozygosity on angiogenesis, showing the existence of a gene titration effect.

The mRNA contents of VEGF-A and eNOS were reduced in the ischemic muscles of diabetic mice, thus supporting the possibility that the leptin signalling may be important for the native modulation of angiogenic effectors. This is in keeping with previous observations showing that leptin increases eNOS phosphorylation in Ser<sup>1177</sup> and stimulates NO release by cultured ECs and isolated vessels (32). However, diabetes-induced endothelial dysfunction and/or microvessel rarefaction, independent of the leptin knock-out, may, per se, have contributed to the expression changes observed in Lepr<sup>db/db</sup>. Lepr<sup>db/+</sup> showed a mildly reduced haemodynamic recovery and impaired ischemia-induced eNOS up-regulation. In apparent contradiction with the finding of Schiekofer et al (17), VEGF-A was not up-regulated by ischemia in our wild type mice. However, the time points chosen by Schiekofer and colleagues to perform mRNA analyses differ from ours. We worked at 3 days post-ischemia. The other group worked at 1, 7, and 14 days thereafter. It is noteworthy that they could find ischemia-induced VEGF-A mRNA upregulation only at 7 days or later. As angiogenesis is a phenomenon which develops early after ischemia and increases in capillary density are already appreciable after 7 days (18), the probability that gene expression changes at 7 days account for post-ischemic angiogenesis may be considered scarce. On the other hand, in agreement with previous findings that endogenous eNOS is essential for post-ischemic angiogenesis (35,36), we found increased eNOS mRNA levels in the ischemic muscles of wild type mice.
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Figure 7. A. Realtime RT-PCR results of human TK (A and B) and 18S rRNA (C and D) analyses of Lepr\textsuperscript{db/db} muscles injected with Ad.hTK (hTK) or Ad.Luc (Luc). Amplification (A and C) and melting curves (B and D) are shown. No product was amplified in Ad.Luc-injected muscles. In B, * indicates formation of primer dimers in the Ad.Luc group, while in the Ad.hTK-injected samples, hTK was amplified. B. Western blot band for phosphorylated AktS473, total Akt, and the housekeeping protein GAPDH in extracts from Lepr\textsuperscript{db/db} ischemic muscles which were preliminarily (3 days) infected with Ad.Myr-Akt or the control virus Ad.Luc. The increased content of both phosphorylated AktS473 and total Akt is evident in Ad.Myr-Akt–given muscles.

Figure 8. Reparative angiogenesis in ischemic muscles of Lepr\textsuperscript{db/+} (A) and Lepr\textsuperscript{db/db} (B) is improved by local delivery of adenoviruses carrying the genes of tissue kallikrein (TK) or the active form of Akt (Myr-Akt). Lepr\textsuperscript{db/+} and Lepr\textsuperscript{db/db} controls received an adenovirus carrying luciferase gene (Luc). Mice (n=8 per group) were submitted to left limb ischemia immediately before receiving gene transfer in the ischemic adductor. Two weeks thereafter, ischemic (I) and contralateral (C) adductors were harvested for histological examination of capillary density. Values are mean±SEM. "P<0.01 and "P<0.05 vs C; °P<0.05 vs Luc.

and, to a lesser extent, Lepr\textsuperscript{db/+}. By contrast, ischemia did not increase eNOS mRNA in Lepr\textsuperscript{db/db}. As elegantly demonstrated by the Dimmeler group, eNOS is under the transcriptional control of FOXO-1 and FOXO-3 and active FOXOs impair angiogenesis and repress eNOS expression. (37) As FOXO became inactivated following its
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Figure 9. Effects of gene transfer with Ad.hTK (hTK, red) or Ad.Myr.Akt (Akt, blu) or Ad.Luc (Luc) to the post-ischemic blood flow recovery of Leprdb/+ (A) and Leprdb/db (B). The recovery of BF to the ischemic foot was measured by Laser Doppler Flowmetry. It is evident that angiogenesis gene therapy translated into improved BF recovery in Leprdb/+. By contrast, the same approaches were ineffective in the Leprdb/db. Values are mean±SEM. °P<0.05 vs Luc.

phosphorylation by Akt (38), it is possible that an impaired Akt activity may contribute to reduced eNOS level observed in the ischemic muscles of Leprdb/db. Consistently, gene therapy with constitutionally active Akt restored the proper angiogenesis response to ischemia in the diabetic mice. Nevertheless, blood flow recovery was still compromised. This suggests that the defective reparative neovascularization is not the sole responsible for the impaired post-ischemic recovery of Leprdb/db.

An important finding of this study consists of the unexpected result that angiogenesis gene therapy with either hTK or constitutively activated Akt failed to improve the blood flow recovery in the Leprdb/db. This failure occurred despite the potent pro-angiogenic and pro-arteriogenic effects exerted by either gene transfer approach.

We have previously shown that intra-muscular hTK gene transfer, exert a potent therapeutic effect in the limb muscles of normoglycemic or type-1 diabetic mice with peripheral ischemia (9,18). One of the kallikrein products, bradykinin activates eNOS via the PI3K-Akt and calcineurin pathways (39). In vivo, we showed that hTK activates Akt-NO pathway by a VEGF-independent mechanism (19). Moreover, Akt gene transfer was previously shown to induce therapeutic angiogenesis and to ameliorate the BF to ischemic limbs (27). In this study, gene transfer was performed correctly, as demonstrated by the presence of mRNA for the human form of TK in Ad.hTK-injected ischemic muscle of Leprdb/db and by increased total and phosphorylated Akt protein in Ad.Myr.Akt-given diabetic muscles. Moreover, either Ad.hTK or Ad.Myr.Akt produced angiogenesis and normalized VEGF-A and eNOS expression in ischemic muscles of Leprdb/db; thus further supporting proper experimental procedures. More importantly, in non obese and non diabetic heterozygous mice, the same gene therapies did improve blood flow recovery. Having discounted for technical problems, the other possible explanation of the negative results observed in Leprdb/db would be the intrinsic unresponsiveness to pro-angiogenesis treatment as the sole way to address vascular liabilities. Specifically, although numerically augmented, the newly generated vessels of diabetic mice might be dysfunctional, as recently reported by our group (42) and prone to thrombosis. In addition, diabetic myopathy could be refractory to de novo neovascularization.

In conclusion, our results suggest that stimulation of neovascularization might not suffice as a sole therapeutic avenue to combat type-2 diabetes-related vascular complications. Therapeutic angiogenesis might represent a complement, rather than an alternative, to a global treatment strategy including extreme revascularization, anti-oxidant agents, and metabolic control of hyperglycemia for the cure of diabetes complications. Consequently, angiogenesis gene therapy needs further preclinical optimization in appropriate animal models to improve the chances of clinical success.

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7. REFERENCES

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