[Frontiers in Bioscience 12, 2003-2012, January 1, 2007]

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

Costanza Emanueli[1,2], Andrea Caporali[1], Nicolle Krankel[1,2], Brunella Cristofaro[1], Sophie Van Linthout[2,4], Paolo Madeddu[1,2,3]

[1]The Bristol Heart Institute, University of Bristol, Bristol, UK; [2]The National Institute of Biostructures and Biosystems (INBB), Alghero and Osilo, Italy; [3]Multimedica Hospital, Milan, Italy; [4]Charite, University Medicine Berlin, Campus Benjamin Franklin, Department of Cardiology, Berlin, Germany

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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$^{db/db}$ mice (*Lepr$^{db/db}$*), and in non-diabetic heterozygous *Lepr$^{db/+}$*. 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$^{db/db}$* and *Lepr$^{db/+}$*, 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$^{db/db}$*, apoptosis of endothelial cells was enhanced and the densities of capillary and arteriole were reduced. Arterioles of *Lepr$^{db/db}$* showed hypertrophic remodelling and, occasionally, lumen occlusion. Following ischemia, *Lepr$^{db/db}$* showed a defective reparative angiogenesis in ischemic muscle, delayed blood flow recovery, and worsened clinical outcome as compared with controls. Five-month old *Lepr$^{db/+}$* displayed an increase in endothelial cell apoptosis under basal conditions, while capillary and arteriole densities were normal. *Lepr$^{db/+}$* 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$^{db/+}$* and *Lepr$^{db/db}$*. However, in the *Lepr$^{db/db}$* 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$^{db/+}$*. 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 completed 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 arterial 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-Lepr$^{db/db}$ mice (*Lepr$^{db/db}$*). We also investigated the angiogenesis phenotype of heterozygous mice (*Lepr$^{db/+}$*). *Lepr$^{db/db}$* carry a genetic mutation of the leptin receptor, which inactivates the receptor signalling. *Lepr$^{db/db}$* develop severe diabetes associated with hyperphagia, obesity, and hyperinsulinemia (11), while *Lepr$^{db/+}$* have a normal phenotype. Although a genetic mutation similar to that of *Lepr$^{db/db}$* 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). *Lepr$^{db/db}$* 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 *Lepr$^{db/db}$* and *Lepr$^{db/+}$* and the angiogenesis and apoptosis responses to peripheral ischemia in 5-month old *Lepr$^{db/db}$* and *Lepr$^{db/+}$*. 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 pro-angiogenic 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 *Lepr$^{db/db}$* and *Lepr$^{db/+}$*.

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-Lepr$^{db/db}$ mice (*Lepr$^{db/db}$*, Harlan, Milan, Italy) and non-diabetic *Lepr$^{db/+}$* 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 anaesthetized (2,2,2-tribromoethanol –avertin-, 880 mmol/kg body wt IP, Sigma-Aldrich, Italy) *Lepr$^{db/db}$*, *Lepr$^{db/+}$*, 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 autoamputation rate and avoid sufferance to the animals (9). In another experiment, 5-month old *Lepr$^{db/db}$* and *Lepr$^{db/+}$* were submitted to ischemia and injected with one of the adenoviruses ($1x10^8$ 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 *Lepr$^{db/db}$* was verified. Realtime RT-PCR was performed on extracts from *Lepr$^{db/db}$* ischemic muscles injected with *Ad.hTK* or *Ad.Luc* to evaluate the human tissue kallikrein transgene expression using specific primer pairs (*hTK* sense: 5'-TGACAGAGCCTGCTGATACC-3', *hTK* antisense: 5'-TCACCCACACAGGTGTCTTT-3') and the LightCyclerTM technology (RocheDiagnostics, UK). The amount of *hTK* mRNA was normalized to the amount

Table 1. Body Weight and Hemodynamic Parameters

	WT	*Lepr*$^{db/+}$	*Lepr*$^{db/db}$
BW (g), 3 months	23±1	28±2*	52±2**§§
5 months,	29±2	30±2	55±2**§§
BP (mmHg), 3 months	109±4	106±6	105±11
5 months	122±4	128±12	126±12
HR (b/min), 3 months	698±14	528±52*	529±50*
5 months	675±14	515±87*	553±55*
Glucosuria, 3 months	Absent	Absent	Present
5 months	Absent	Absent	Present

Values are means±SEM. Each group consisted of 8 mice. BW, Body Weight; BP, Tail-Cuff Blood Pressure; HR, Heart Rate. *P<0.05 and **P<0.01 vs. WT; §§P<0.01 vs. *Lepr*$^{db/+}$

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.

3.5. Quantitavive PCR for Measuring Muscular eNOS and VEGF-A content

Real-time quantitative PCR (ABI PRISM® 7000 Sequence Detection System software version 1.0., Perkin Elmer) with internal standards was used to determine *eNOS* and *VEGF-A* mRNA content in ischemic (at 3 days post-ischaemia induction) and contralateral muscles of *Lepr*$^{db/db}$, *Lepr*$^{db/+}$, and WT (n=4 to 5 mice per group). We also measured the mRNA levels of *eNOS* and *VEGF-A* in ischemic muscles of *Lepr*$^{db/db}$ which had received *Ad.hTK*, *Ad.Myr-Akt*, or *Ad.Luc* (n=5 mice per group). *ENOS* and *VEGF-A* were normalized to *GAPDH* levels. All procedures (including PCR primers and internal standards) have been described previously (19).

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 auto-amputation 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 P value <0.05 was interpreted to denote statistical significance.

4. RESULTS

4.1. Cardiovascular Phenotype of *Lepr*$^{db/db}$

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

4.2. Microangiopathy of *Lepr*$^{db/db}$

In limb muscles of 3- and 5-month old *Lepr*$^{db/db}$, capillary density was lower than in WT (P<0.01 for both comparisons) (Figure 1A). Similarly reduced (P<0.01) was the capillary to myofiber ratio (Figure 1B). *Lepr*$^{db/+}$ 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*$^{db/db}$ (9.1±1.1 vs. 23.0±2.1 art/mm^2 in WT at 3 months: 8.1±1.6 vs. 22.6±1.4 art/mm^2 in WT at 5 months, p<0.01 for both comparisons). In *Lepr*$^{db/db}$, microvessel rarefaction was associated with increased EC apoptosis (Figure 1D). In *Lepr*$^{db/db}$, apoptosis was also activated at myofiber level (26±4 vs 6±1 TUNEL-positive myofibers/mm^2 in WT, P<0.01, Figure 1D). In *Lepr*$^{db/+}$, 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<0.01 vs WT, Figure 1D). Capillary and arteriole density was normal in the muscles of 3- and 5-month old *Lepr*$^{db/+}$.

4.3. Impaired Reparative Angiogenesis in *Lepr*$^{db/db}$

We then examined the ability of 5-month old *Lepr*$^{db/db}$ and *Lepr*$^{db/+}$ 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^2 in contralateral muscle, P<0.01). A similar increment was observed in *Lepr*$^{db/+}$ (1036±54 vs 752±49 cap/mm^2 in contralateral, P<0.01). In contrast, *Lepr*$^{db/db}$ failed to mount reparative capillarization (621±14 vs. 570±19 cap/mm^2 in contralateral muscle, P=N.S.). This deficit was also evident when considering the capillary to myofiber ratio of *Lepr*$^{db/db}$ ischemic muscles (0.88±0.03 vs 1.58±0.08 in *Lepr*$^{db/+}$ and 1.66±0.07 in WT, P<0.01 for both comparisons).

In ischemic muscles of *Lepr*$^{db/db}$, arteriole number was less (10.5±1.8 art/mm^2) than in WT (24.6±2.9 art/mm^2, p<0.05) or *Lepr*$^{db/+}$ (24.8±2.4 art/mm^2, P<0.05). Arteriole density of *Lepr*$^{db/+}$ was normal (P=NS vs WT). As shown in Figure 3, EC apoptosis was higher in the ischemic adductors of *Lepr*$^{db/db}$ (55±12 TUNEL-positive EC/mm^2) than in WT (8±1 TUNEL-positive EC/mm^2, P<0.01). EC apoptosis was also elevated in *Lepr*$^{db/+}$ (28±10 TUNEL-positive EC/mm^2 , P<0.05 vs. WT). Myocyte apotosis was increased in the ischemic muscles f *Lepr*$^{db/db}$, only (27±10 vs 6±1 TUNEL-positive myocytes/mm^2 in WT, P<0.05).

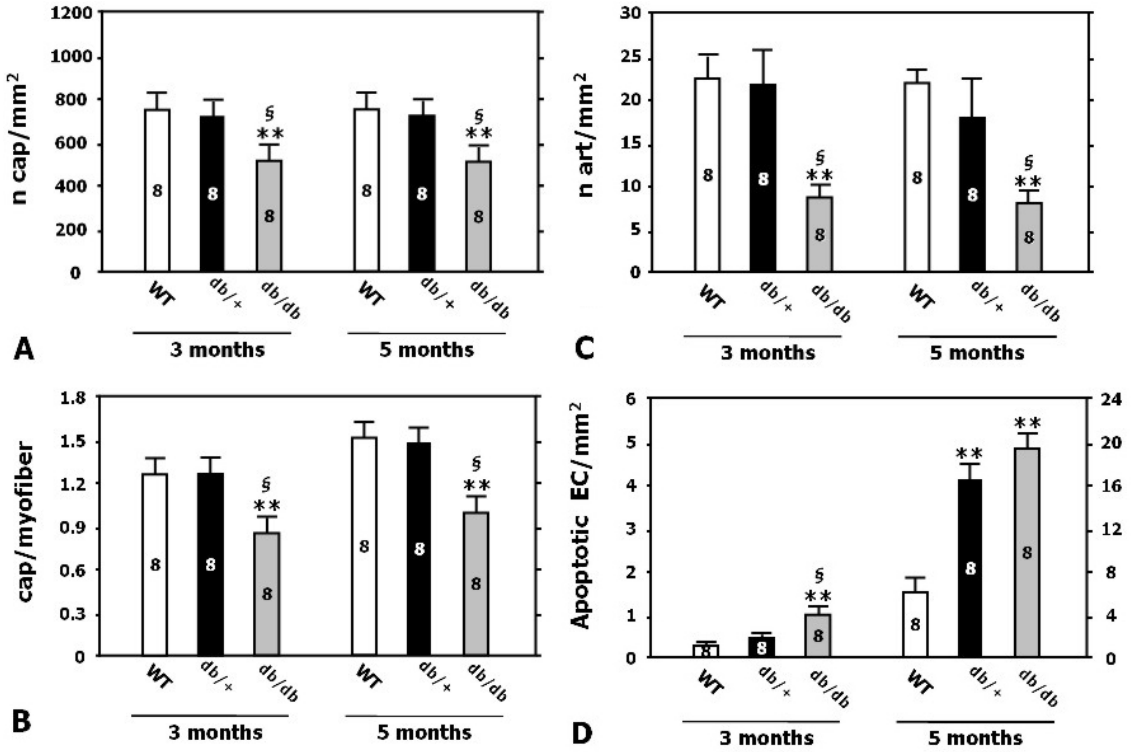

Figure 1. Time course of the changes in capillary (cap) density (A), capillary to myofiber ratio (B), arteriole (art) density (C), and endothelial cell apoptosis (D) in adductor muscles from *Lepr*$^{db/db}$ (db/db, grey columns) and *Lepr*$^{db/+}$ (db/+, black columns). Values in age-matched wild-type 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*$^{db/+}$.

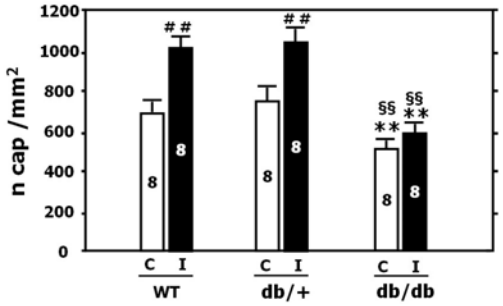

Figure 2. Reparative angiogenesis is impaired in ischemic muscles of *Lepr*$^{db/db}$. Capillary density of ischemic (I) and contralateral (C) adductors was analyzed at 2 weeks from ischemia induction. Values are mean±SEM and number within each column represents sample size. $^{\#\#}$*P*<0.01 vs C; ***P*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

As shown in Figure 4, arterioles in ischemic muscles of *Lepr*$^{db/db}$ 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*$^{db/db}$

As shown in Figure 5A, BF recovery was significantly delayed in *Lepr*$^{db/db}$ (P<0.05 vs. WT). The trend observed in *Lepr*$^{db/+}$ 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*$^{db/db}$ 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*$^{db/db}$ (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*$^{db/db}$

As shown in Figure 6, the *VEGF-A* mRNA level was 1.9-fold lower in ischemic muscles of *Lepr*$^{db/db}$ compared with WT (P<0.05). *VEGF-A* expression was

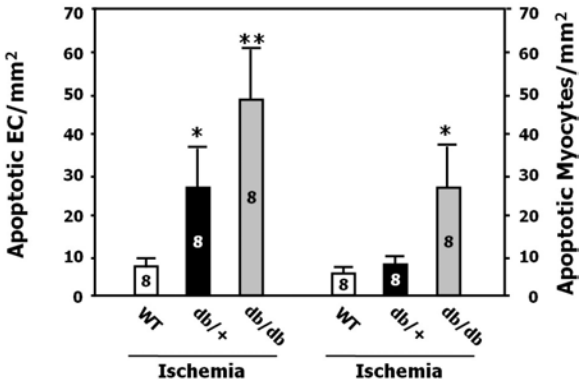

Figure 3. Endothelial cell (EC) apoptosis (left side) is increased in the ischemic adductor muscles of *Lepr*$^{db/db}$ and *Lepr*$^{db/+}$. Myocyte apoptosis (right side) is increased in *Lepr*$^{db/db}$, only. Apoptosis was determined by in situ TUNEL staining of ischemic muscles harvested at 2 weeks post-ischemia. Values are mean±SEM and number within each column represents sample size. ***P*<0.01 and **P*<0.05 vs WT.

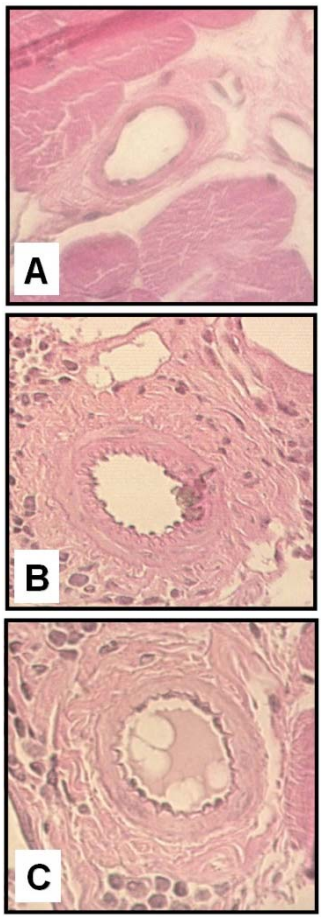

Figure 4. Representative HE-stained sections (captured at 400 X) from ischemic adductors of WT (A) and *Lepr*$^{db/db}$ (B, C). Arterioles of diabetic mice are characterized by wall thickening (B) and lumen occlusion (B), and they are surrounded by monocytes infiltrating the interstitial space (A, B).

reduced by 1.4-fold in *Lepr*$^{db/+}$, 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*$^{db/+}$ (2.0-fold increase vs contralateral, P<0.05). In contrast, the modulation of *eNOS* by ischemia was abrogated in *Lepr*$^{db/db}$.

4.6. Effects of Angiogenesis Gene Therapy in *Lepr*$^{db/db}$ and *Lepr*$^{db/+}$

As shown in Figure 7A, *hTK* mRNA was present in *Ad.hTK*-injected *Lepr*$^{db/db}$ 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*$^{db/+}$ (Panel A) and *Lepr*$^{db/db}$ (Panel B). Moreover, both *Ad.hTK* and *Ad.Myr-Akt* increased arteriole density in ischemic muscles of *Lepr*$^{db/db}$ (*Ad.hTK*: 37±6 art/mm^2; *Ad.Myr.Akt*: 21±3 art/mm^2, P<0.05 for both comparisons vs *Ad.Luc*: 12±7 art/mm^2) and *Lepr*$^{db/+}$ (*Ad.hTK*: 32±9 art/mm^2; *Ad.Myr.Akt*: 22±6 art/mm^2, P<0.05 for both comparisons vs *Ad.Luc*: 10±6 art/mm^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*$^{db/+}$, *hTK*- or *Akt*-induced neovascularization translated into improved recovery of BF to the ischemic muscles (see Figure 9A). In strict contrast, in *Lepr*$^{db/db}$, 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* $^{db/db}$ 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*$^{db/+}$ showed a milder phenotype, with EC apoptosis being activated at later stages.

The capillary rarefaction that we have observed in *Lepr* $^{db/db}$ 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

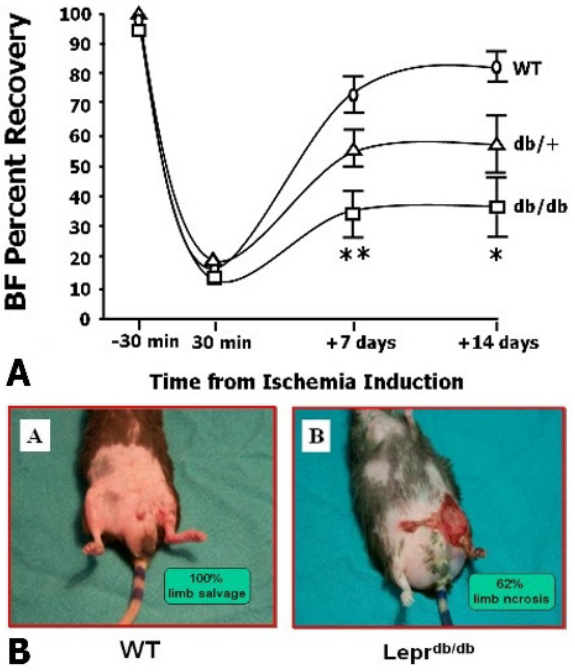

Figure 5. A: Blood flow (BF) recovery following induction of unilateral limb ischemia in *Lepr*$^{db/db}$ (open squares), *Lepr*$^{db/+}$ (open triangles), and WT (open circles). The recovery of superficial limb blood, measured by Laser Doppler Flowmetry, was delayed in *Lepr*$^{db/db}$. Values are mean±SEM. $^{**}P<0.01$ and $^{*}P<0.05$ vs WT. B: Representative images of post-ischemic limbs in WT (A, left) and *Lepr*$^{db/db}$ (B, right). Pictures were taken at 2 weeks from ischemia induction. *Lepr*$^{db/db}$ showed delayed healing of surgical wounds and limb necrosis was a frequent clinical feature in these diabetic mice.

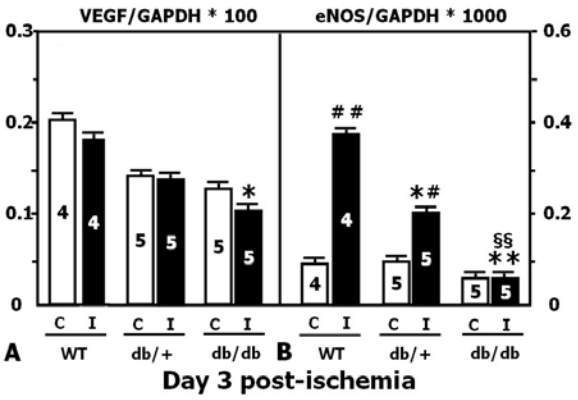

Figure 6. *VEGF-A* (A) and *eNOS* (B) mRNA levels are reduced in ischemic muscles of *Lepr*$^{db/db}$. Muscles were harvested 3 days after ischemia induction and gene expression was determined by quantitative RT-PCR. Values were then normalized by *GAPDH.* Values are mean±SEM and number within each column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

observed in *Lepr*$^{db/db}$. Detrimental haemodynamic effects and tissue hypoxia may derive from the rarefaction of arterioles, the conduit vessels that normally provide the largest part of tissue blood flow. Thus, the microangiopathy observed in *Lepr*$^{db/db}$ recalls the structural alterations described previously in type-2 diabetic patients (34). The observation that microvascular alterations were associated with normal blood pressure levels discount a possible influence of haemodynamic factors on microangiopathy. However, we cannot exclude that, as a consequence of vascular rarefaction and remodeling causing an increase in peripheral vascular resistances, hypertension may develop in elderly *Lepr*$^{db/db}$.

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*$^{db/db}$ 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*$^{db/db}$ 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 arterial remodeling, intra-luminal thrombosis, and uniquely activated apoptosis in vascular and muscular cells of *Lepr*$^{db/db}$ 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^{1177} 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 expressional changes observed in *Lepr*$^{db/db}$. *Lepr*$^{db/+}$ 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 expressional 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

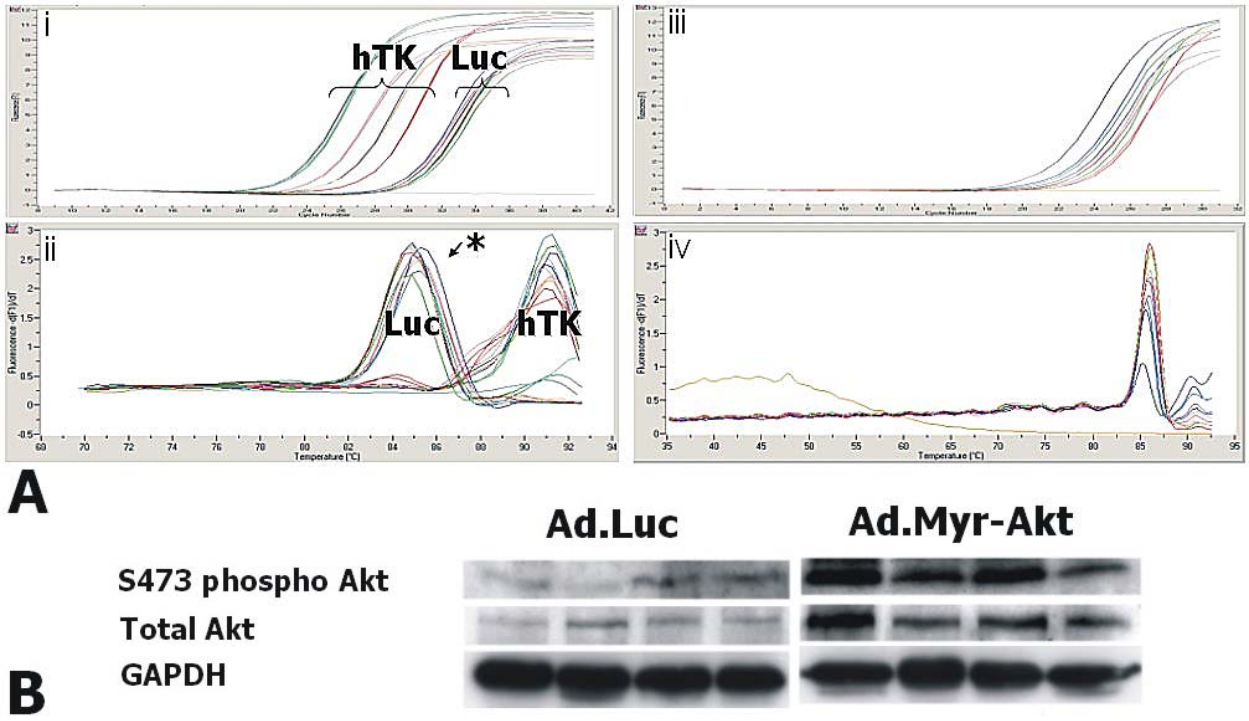

Figure 7. A. Realtime RT-PCR results of human *TK* (A and B) and 18S rRNA (C and D) analyses of *Lepr*$^{db/db}$ muscles injected with *Ad.hTK* (hTK) or *Ad.Luc* (Luc). Amplification (A and C) and melting curves (B and D) are shown. No product was amplified in *Ad.Luc*-injected muscles. In B, * indicates 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*$^{db/db}$ 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.

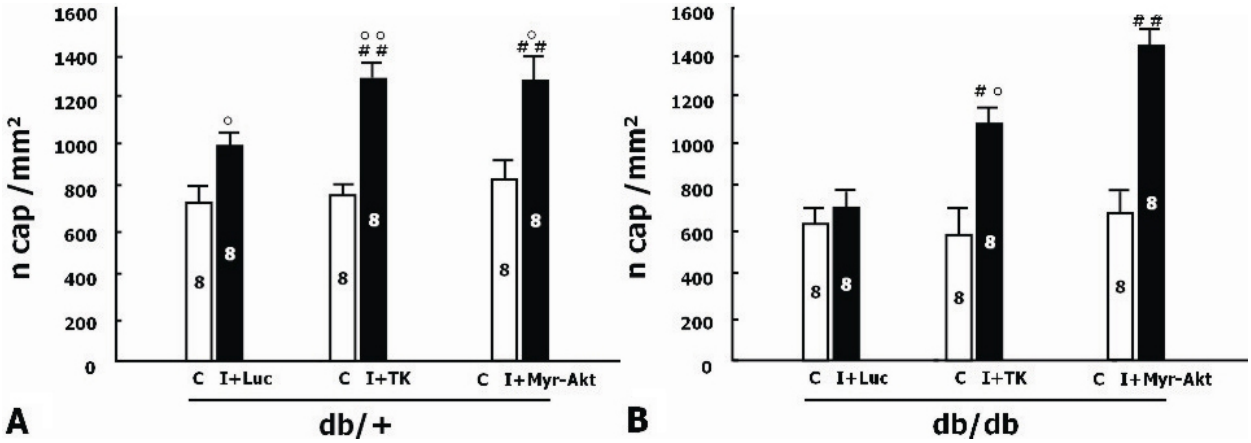

Figure 8. Reparative angiogenesis in ischemic muscles of *Lepr*$^{db/+}$ (A) and *Lepr*$^{db/+db}$ (B) is improved by local delivery of adenoviruses carrying the genes of tissue *kallikrein* (TK) or the active form of *Akt* (Myr-Akt). *Lepr*$^{db/+}$ and *Lepr*$^{db/db}$ controls received an adenovirus 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; $^{\circ}P<0.05$ vs *Luc*.

and, to a lesser extent, *Lepr*$^{db/+}$. By contrast, ischemia did not increase *eNOS* mRNA in *Lepr*$^{db/db}$. 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

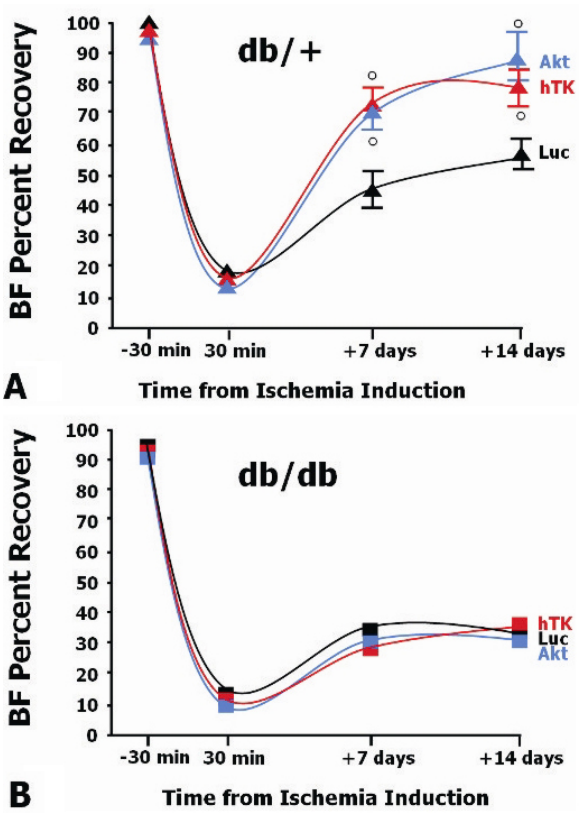

Figure 9. Effects of gene transfer with *Ad.hTK* (hTK, red) or *Ad.Myr.Akt* (Akt, blu) or *Ad.Luc* (Luc) to the post-ischemic blood flow recovery of $Lepr^{db/+}$ (A) and $Lepr^{db/db}$ (B). The recovery of BF to the ischemic foot was measured by Laser Doppler Flowmetry. It is evident that angiogenesis gene therapy translated into improved BF recovery in $Lepr^{db/+}$. By contrast, the same approaches were ineffective in the $Lepr^{db/db}$. 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 $Lepr^{db/db}$. 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 $Lepr^{db/db}$.

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 $Lepr^{db/db}$. 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 $Lepr^{db/db}$ 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 $Lepr^{db/db}$, 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 $Lepr^{db/db}$ 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.

6. ACKNOWLEDGMENTS

This work was supported by a grant from the European Foundation for the Study of Diabetes (EFSD) and Servier to Prof. Madeddu and Dr. Emanueli. Prof. GL. Condorelli (University of Rome La Sapienza, Italy) provided *Ad.Myr-Akt*. Dr. Emanueli holds a British Heart Foundation (BHF) Basic Science Lectureship. Andrea Caporali is a BHF PhD student. Dr. Nicolle Krankel is supported by a Marie Curie grant for the transfer of knowledge granted to Prof. Paolo Madeddu. We thank Mrs Jan Wild for the revision of the English style of the manuscript.

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Key Words: Type-2 diabetes, Leprdb/db mice, Angiogenesis, Apoptosis, Limb Ischemia, Gene Therapy, Tissue Kallikrein, Akt, eNOS

Send correspondence to: Dr. Costanza Emanueli, Bristol Heart Institute, Bristol Royal Infirmary-level 7, University of Bristol, Upper Maudlin Road, BS2 8HW, Bristol, UK, Tel: 4401179283512, Fax: 4401179283904, E-mail: mdxce@bristol.ac.uk

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