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Research Article

Adiponectin Fails in Improving Angiogenic Repair in Streptozocin-Treated or Lepr$^{db/db}$ Mice after Hind Limb Ischemia

Kurt Belisle,[1] Martin Andrassy,[2] Jochen Schneider,[3,4] and Stephan Schiekofer[1]

[1] *Center for Geriatric Medicine, Bezirksklinikum Regensburg, Universitätsstraße 84, 93053 Regensburg, Germany*
[2] *Department of Internal Medicine III, Heidelberg University, 69117 Heidelberg, Germany*
[3] *Translational & Experimental Medicine, Luxembourg Centre for Systems Biomedicine, Luxembourg*
[4] *Department of Medicine II, Saarland University, Medical Center, 66421 Homburg, Germany*

Correspondence should be addressed to Stephan Schiekofer, stephan.schiekofer@medbo.de

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Objectives. Type 1 and 2 diabetes carry risk factors for the development of microvascular diseases with associated impairment of angiogenic repair. Here, we investigated whether adiponectin, an adipocyte-specific adipocytokine with antiatherosclerotic and antidiabetic properties, regulates angiogenic repair in response to tissue ischemia in Lepr$^{db/db}$ and streptozocin-treated diabetic mouse models. *Methods*. Adenoviral vectors containing the gene for β-galactosidase, full-length mouse adiponectin, and dominant-negative AMPKα2 were used in streptozocin-treated male Lepr$^{db/db}$ mice, after which hind limb blood flow was measured using a laser doppler blood flow analyzer. *Results*. The angiogenic repair of ischemic hind limbs was impaired in both streptozocin-treated and Lepr$^{db/db}$ mice compared to wild-type mice as evaluated by laser doppler flow and capillary density analyses. Adenovirus-mediated administration of adiponectin accelerated angiogenic repair after hind limb ischemia in WT mice, but not in Lepr$^{db/db}$ mice or mice treated with streptozocin. In vitro experiments using HUVECs highlighted the antiapoptotic and proangiogenic properties of adiponectin but could not demonstrate accelerated differentiation of endothelial cells into tube-like structures at elevated glucose levels. *Conclusions*. External administration of adiponectin at elevated glucose levels may not be useful in the treatment of diabetes mellitus-related vascular deficiency diseases.

1. Introduction

Type 1 diabetes is caused by absolute insulin deficiency, whereas glucose intolerance and peripheral insulin resistance characterize Type 2 diabetes. Type 2 diabetes often occurs in patients displaying a combination of clinical and biochemical symptoms [1–4]. Both types of diabetes are associated with microvascular rarefaction and reduced collateralization in ischemic tissues. These circulatory deficits often lead to ischemic injury and impaired wound healing. However, the mechanisms by which diabetes inhibits angiogenic repair in ischemic tissues are poorly understood.

Adiponectin/ACRP30 is a circulating adipocyte-derived cytokine whose levels are decreased in patients with obesity, diabetes, and coronary artery diseases [5–8]. Adiponectin is known to promote insulin sensitivity by decreasing triglyceride content in muscle and liver in obese mice [9–13]. Triglycerides are reduced primarily through the regulation of enzymes involved in both fatty-acid combustion and energy dissipation. Adiponectin reduces atherosclerotic lesions in apolipoprotein E-deficient mice and has anti-inflammatory effects on the vasculature [14–16]. It has been shown that adiponectin acts upon endothelial cells to promote angiogenesis and that ischemic-induced neovascularization is impaired in adiponectin-deficient mice [17, 18]. Adiponectin reportedly also protects endothelial cells from apoptosis [19, 20]. Nevertheless, the administration of adiponectin for therapeutic angiogenic repair in the context of diabetes-related vascular deficiency has not been evaluated.

In this study, we investigated whether adiponectin modulates the angiogenic recovery process in the ischemic hind limbs of streptozocin-treated and Lepr$^{db/db}$ mice.

Insulin deficiency was induced by intraperitoneal administration of the β-cell toxin streptozocin (db1 mice) [21, 22]. The mouse model for Type 2 diabetes employed the db/db mouse strain, a strain that lacks a functional leptin receptor [23]. These mice become identifiably obese at 3 to 4 weeks of age [24–26]. Elevation of plasma insulin begins at 10 to 14 days and of blood sugar at 4 to 8 weeks. These mice are polyphagic, polydipsic, and polyuric.

Here we show that when compared to control mice, both mouse models of diabetes have impaired angiogenic repair following hind limb ischemic surgery. In both diabetes models adiponectin treatment was evaluated for its ability to stimulate angiogenic repair. Our observations indicate that administration of adiponectin may not be instrumental in the treatment of vascular deficiency disease associated with chronic hyperglycemia.

2. Materials and Methods

2.1. Materials. Recombinant human vascular endothelial growth factor (VEGF) was purchased from Sigma (St. Louis, MO, USA). The use of recombinant adiponectin, adenovirus vectors containing the gene for β-galactosidase (Ad-βgal), full-length mouse adiponectin (Ad-APN), and dominant-negative AMPKα2 (Ad-dnAMPK) was described previously [17, 27, 28].

2.2. Mouse Models

Streptozocin-Treated Mice. Diabetes was induced by intraperitoneal injection of STZ (= streptozocin) in male wild-type mice in a C57/BL6 background at 4 weeks of age. Streptozocin was administered at 60 mg/kg, freshly dissolved in 0.05 M sterile sodium citrate, pH 4.5 on 6 subsequent days [21, 22]. Control animals received sodium citrate only. Diabetes was verified 16–25 days later by measuring blood glucose levels in samples drawn from the tail vein using blood glucose test sticks and a conventional glucometer (Hypoguard, Minneapolis, Minnesota, USA).

In the first 2 weeks after onset of diabetes blood glucose levels were measured daily. If glucose levels occasionally recovered, an additional STZ injection was given on days 25–27. More than 90% of mice became diabetic within the first 4 weeks. Insulin injection started as soon as blood glucose increased above 300 mg/dL and was administered as individual injection of 1-2 units of Insulin Ultralente (100 U/mL; Lilly, Indianapolis, USA). After blood glucose levels stabilized, blood glucose levels were measured at a minimum of once a week. Despite insulin replacement, the mice remained hyperglycemic with serum glucose values >200 mg/dL. The outcomes observed in STZ-treated C57/BL6 mice were compared with untreated C57/BL6 mice.

Lepr$^{(db/db)}$ Mice. Male Lepr$^{db/db}$ mice in a C57/BL6 background and male wild-type (WT) mice (C57/BL6) purchased from The Jackson Laboratory were used for this study. The outcomes observed in Lepr$^{db/db}$ mice were compared to C57/BL6 mice as controls.

At the age of 8 weeks, mice were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal) and subjected to unilateral hind limb surgery [29, 30]. An incision was performed in the dermis overlying the middle portion of the left hind limb. After ligation of the proximal end of the femoral artery, the distal portion of the saphenous artery was ligated, and the artery, as well as all side branches, was dissected free and excised. The skin was closed using a surgical stapler. Before surgery, body weight (BW) was determined, and systolic blood pressure (sBP) was determined using a tail-cuff pressure analysis system while mice were in the conscious state.

In some experiments, 2×10^8 plaque-forming units (PFU) of adenoviral vector encoding adiponectin (Ad-APN) or β-galactosidase (Ad-βgal) were injected into the jugular vein of mice 3 days prior to the ischemic hind limb. For adenovirus experiments ischemic/nonischemic laser doppler blood flow (LDBF) ratios were examined at 14 days after surgery, which is compatible with the time course of adenovirus-mediated gene expression.

2.3. Serum Measurement. Blood samples were collected from the tail vein on the day of surgery. Glucose was measured with an enzymatic kit (Wako Chemicals, Richmond, Virginia, USA). Leptin levels were measured with mouse leptin enzyme-linked immunosorbent assay (ELISA) kit (Crystal CHEM INC, Downers Grove, Illinois, USA). Mouse adiponectin levels were determined with an adiponectin ELISA kit (Otsuka Pharmaceutical Co Ltd, Tokyo, Japan). For adiponectin, blood samples were collected from tail vein at the time of hind limb surgery, which was 3 days after the administration of the adenoviral vectors.

2.4. Laser Doppler Blood Flow Analysis. After anesthesia, hair was removed from both legs using a depilatory cream. Mice were then placed on a heating plate at 37°C for 10 minutes to minimize temperature variations. Hind limb blood flow was measured using a laser doppler blood flow (LDBF) analyzer (Moor LDI; Moor Instruments, Devon, United Kingdom). Immediately before surgery, immediately after surgery, and on postoperative days 3, 7, and 14 LDBF analyses were performed on legs and feet. Blood flow was displayed as changes in the laser frequency using different color pixels. After scanning, stored images were analyzed to quantify blood flow. To avoid data variations due to ambient light and temperature, hind limb blood flow was expressed as the ratio of left (ischemic) to right (nonischemic) LDBF.

2.5. Tissue Preparation and Immunohistochemistry. The mice were sacrificed with an overdose of sodium pentobarbital. For immunohistochemistry, muscle samples were imbedded in optimal cutting temperature (OCT) compound (Miles, Elkhart, Indiana, USA) and snap-frozen in liquid nitrogen. Tissue slices (5 μm in thickness) were prepared, and immunohistochemistry was performed using antibodies for CD31 (PECAM-1: Becton Dickinson, Franklin Lakes, New Jersey, USA). Capillary density within the adductor muscle was quantified by histological analysis. Fifteen randomly

chosen microscopic fields from three different sections in each tissue block were examined for the presence of capillary endothelial cells for each mouse specimen. Capillary density was expressed as the number of CD-31-positive features per high power field (×400) and the number of capillaries per muscle fiber.

2.6. Cell Viability Assays. Human umbilical vein endothelium cells (HUVECs) were purchased from Cambrex Bio Science, Walkersville, MD, USA. The CellTiter 96 AQ_{ueous} kit (Promega, Madison, Wi USA) was used to assess cell viability according to the manufacturer's instructions using a non-radioactive cell proliferations assay (MTS) reagent. Briefly, HUVECs were plated at a density of 1.5×10^4 cells/well in a 96-well plate and incubated in growth media for 18 hours. Cells were treated with indicated concentrations of D-glucose (or D-mannitol as osmolarity control) and recombinant adiponectin or 10 ng/mL VEGF in endothelial basal medium-2 (Cambrex Bio Science, Baltimore, USA) containing 0.5% fetal calf serum (FCS) for 48 hours. In some experiments HUVECs were infected with adenoviral constructs encoding β-galactosidase (Ad-βgal) or dominant-negative AMPKα2 (Ad-dnAMPK) at a multiplicity of infection of 50 for 24 h [17, 27, 28, 31]. The 490 nm absorbance was measured after two more 1-hour incubations with MTS. The percentage of cell death was calculated as the decrease in absorbance relative to cells incubated in 20% FCS.

2.7. Tube Formation Assay. Experimental procedures on formation of vascular-like structures by HUVECs on growth factor-reduced Matrigel (BD Biosciences, NJ, USA) were conducted as previously described [31]. Twenty-four-well culture plates were coated with Matrigel according to the manufacturer's instructions. Serum-starved HUVECs were seeded on coated plates at 5×10^4 cells/well in EBM-2 medium (Cambrex, Walkersville, Md. USA) with 0.5% fetal bovine serum containing the indicated concentrations of recombinant adiponectin, D-glucose or (D-mannitol as osmolaric control) or 10 ng/mL VEGF and incubated at 37°C for 48 h. In some experiments HUVECs were infected with adenoviral constructs encoding β-galactosidase (Ad-βgal) or dominant-negative AMPKα2 (Ad-dnAMPK) at a multiplicity of infection of 50 for 24 h [27, 28, 31]. Tube formation was observed using an inverted phase contrast microscope (Nikon, Tokyo, Japan). Images were captured with a video graphic system (DEI-750 CE Digital Output Camera, Optronics, Goleta, CA). The degree of tube formation was quantified by measuring the length of tubes in 3 randomly chosen low power fields (×100) from each well using the National Institutes of Health (NIH) image program (NIH *Image*). Each experiment was repeated 3 times.

2.8. Statistical Analysis. Data are presented as mean ± SE. Statistical analysis was performed by analysis of variance (ANOVA). A value of $P < 0.05$ was accepted as statistically significant.

3. Results

The mice analyzed in this study were male C57/BL6 (WT), C57/BL6 treated with streptozocin (db1), as a model of insulin deficiency, and $Lepr^{db/db}$ on the C57/BL6 background, as a model of metabolic syndrome and Type 2 diabetes (Figure 1(a)). Blood pressure did not differ significantly between the three groups (Table 1) on the day of surgery. Significant differences were observed in body weight, plasma glucose, and leptin levels between WT, db1, and $Lepr^{db/db}$ mice ($n = 6$) (Table 1). Plasma adiponectin levels were significantly elevated in db1 and $Lepr^{db/db}$ mice, relative to WT.

All mice survived the surgical induction of unilateral left hind limb ischemia. After left femoral artery and vein resection, the ratio of blood flow between the ischemic and nonischemic hind limbs decreased to 0.18 ± 0.06 in WT, 0.24 ± 0.04 in db1, and 0.27 ± 0.07 in $Lepr^{db/db}$ mice, indicating that the severity of the induced ischemia was comparable in the three groups. In WT mice, hind limb blood flow perfusion increased to ~60% of the nonischemic limb by day 7 and ultimately returned to ~70% of the nonischemic limb by day 14. In contrast to WT mice, flow recovery in both the db1 and $Lepr^{db/db}$ mice was impaired, and the deficits in flow were statistically significant at the 14th day after surgery (Figures 1(b), 1(c), and 1(d)).

To investigate the extent of vascular remodeling on the microcirculatory level in WT, db1, or $Lepr^{db/db}$ mice, quantitative analysis of capillary density in ischemic and contra lateral adductor muscle of WT, db1, and $Lepr^{db/db}$ mice was determined in histology sections harvested on postoperative day 14. Figure 2(a) shows representative photomicrographs of tissue harvested from WT, db1, or $Lepr^{db/db}$ mice on postoperative day 14 that were immunostained with anti-CD31, an antibody recognizing an endothelial cell marker protein.

Compared with WT, both db1 and $Lepr^{db/db}$ tissue sections appeared to have fewer CD31-positive cells, and these cells appeared to be distributed in a less organized pattern compared with sections from WT animals. Quantitative analysis of CD31-positive cells revealed that the ischemia-induced increase in capillary density of the ischemic limb relative to the contralateral limb was absent in both db1 and $Lepr^{db/db}$ mice (Figure 2(b)), providing further evidence of an impaired angiogenic response in these animals. At the 14-day time point following surgery, the ischemic adductor muscle of both db1 and $Lepr^{db/db}$ mice showed significantly fewer CD31-positive cells compared with ischemic muscle from WT mice.

To investigate whether administration of adiponectin improves vascular remodelling in WT, db1, and $Lepr^{db/db}$ mice, adenoviral vectors expressing either adiponectin (Ad-APN) or β-galactosidase (Ad-βgal) as a control were delivered via jugular vein, 3 days before hind limb surgery in each experimental group. Ad-APN increased plasma adiponectin levels 2.0-fold in WT mice, 1.9-fold in db1 mice, and 1.4-fold in $Lepr^{db/db}$ mice compared to Ad-βgal-treated mice (Table 2). As shown in Figure 3(a), a significant increase in hind limb perfusion was revealed by laser doppler 14 days after hind limb surgery in WT mice that were treated

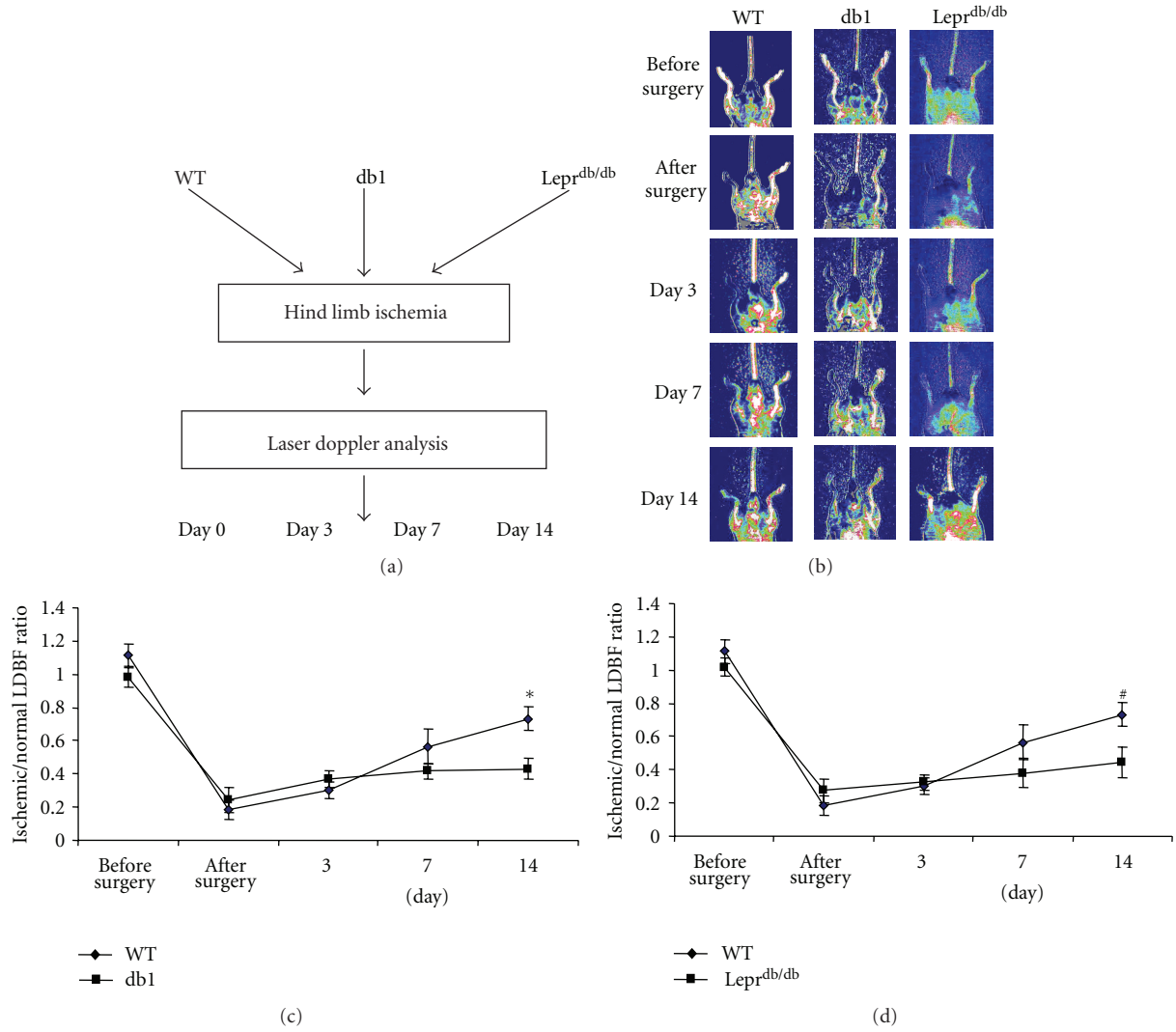

Figure 1: Impaired angiogenic response in the ischemic hind limbs of db1 and Lepr$^{db/db}$ mice. (a) Schematic time course of the study design. (b) A low perfusion signal (dark blue) was observed in the ischemic hind limb of db1 and Lepr$^{db/db}$ mice, whereas a higher perfusion signal (white to red) was detected in WT mice on postoperative days 3, 7, and 14. (c) Quantitative analysis of the ischemic/nonischemic LDBF ratio in WT and db1 mice before hind limb ischemia surgery, shortly after hind limb ischemia surgery, and on postoperative days 3, 7, and 14 ($n = 6$). $^{*}P < 0.01$. (d) Quantitative analysis of the ischemic/nonischemic LDBF ratio in WT and Lepr$^{db/db}$ mice before hind limb ischemia surgery, shortly after hind limb ischemia surgery, and on postoperative days 3, 7, and 14 ($n = 6$). $^{\#}P < 0.05$.

Table 1: Characteristics of WT, db1, and Lepr$^{db/db}$ mice.

Mouse strain	BW	sBP	PG	APN	LP
WT	25.6 ± 0.9	94.2 ± 6.2	142 ± 22	7.1 ± 2.1	2560 ± 336
db1	19.0 ± 0.9	103.8 ± 17.6	426 ± 32	15.9 ± 2.4	561 ± 391
Lepr$^{db/db}$	28.2 ± 1.7	96.5 ± 8.8	230 ± 21	17.0 ± 1.1	45957 ± 4090

Measurements were made on the day of surgery in mice at the age of 8 weeks that were fasted for 6 h ($n = 6$). Each value is means ± S.E. BW indicates body weight (g); sBP: systolic blood pressure (mmHG); PG: plasma glucose (mg/dL); APN: adiponectin (μg/mL); LP: leptin (pg/mL).

with Ad-APN in comparison to control mice (both Ad-βgal-treated and no adenovirus groups). In contrast, db1 mice and Lepr$^{db/+}$ mice showed no significant improvement in hind limb perfusion following administration of adiponectin (Figure 3(a)).

Quantitative analysis of capillary density in ischemic adductor muscle of WT, db1, and Lepr$^{db/db}$ mice was determined in histological sections harvested from the ischemic tissues for each experimental group on postoperative day 14 (Figure 3(b)). Immunostaining with anti-CD31 revealed

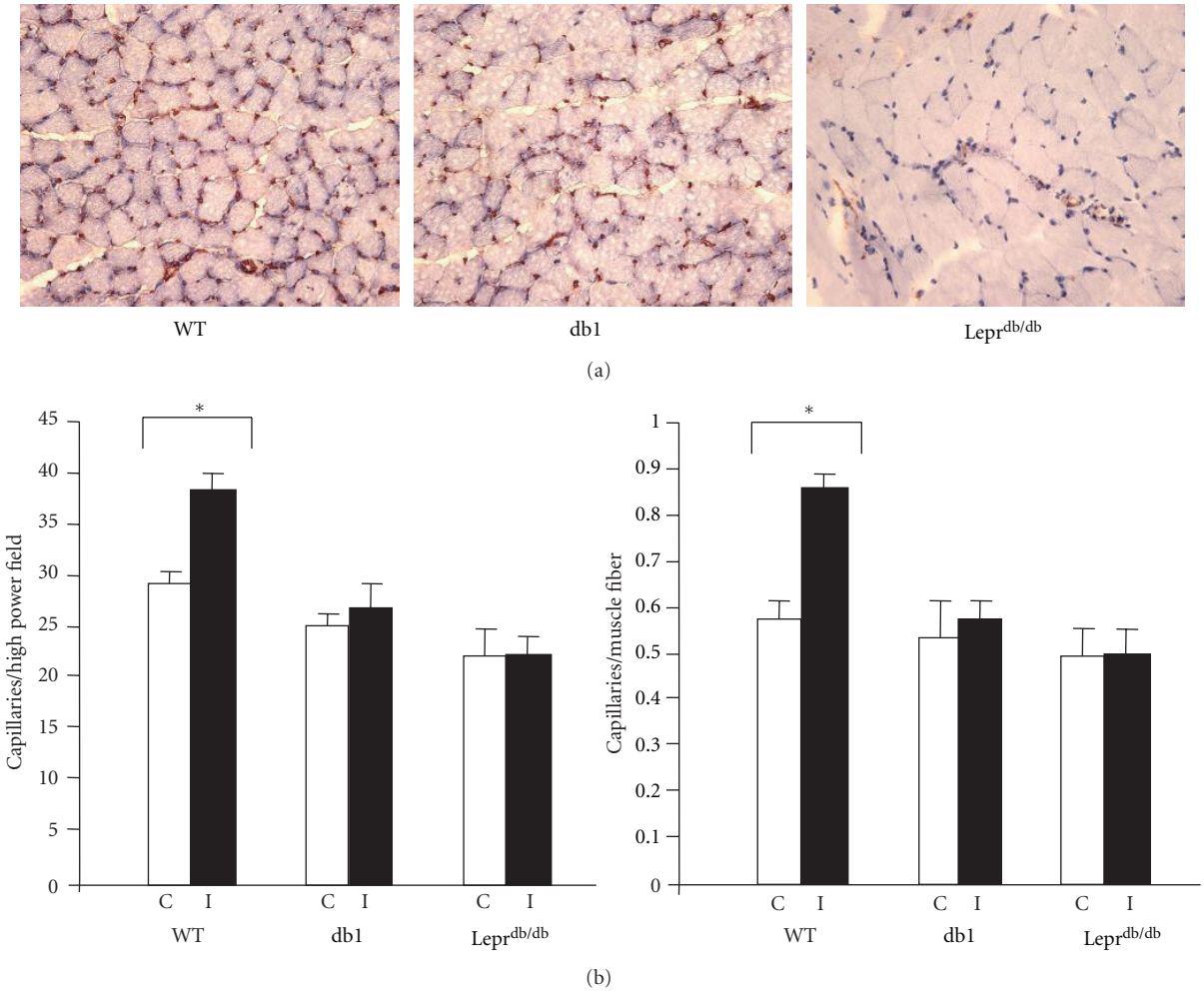

Figure 2: Reduced capillary density in ischemic hind limbs of db1 and Lepr$^{db/db}$ mice. (a) Representative immunostaining of ischemic tissues from WT, db1, and Lepr$^{db/db}$ mice with anti-CD31 monoclonal antibody (brown) on postoperative day 14. (b) Quantitative analysis of capillary density in ischemic (= I) and contralateral (= C) adductor muscle of WT, db1, and Lepr$^{db/db}$ mice on postoperative day 14 ($n = 6$ in each group). Capillary density was expressed as the number of capillaries per high power field (×400, (a)) and capillaries per muscle fiber (b). $^*P < 0.01$.

Table 2: Plasma adiponectin levels in each experimental group.

APN (μg/mL)	3 days after gene transfer		10 days after gene transfer	
Treatment	Ad-βgal	Ad-APN	Ad-βgal	Ad-APN
WT	7.7 ± 0.7	15.2 ± 3.5	8.2 ± 0.5	11.2 ± 1.2
db1	17.3 ± 6.4	29.8 ± 3.8	15.2 ± 3.5	22.7 ± 2.5
Lepr$^{db/db}$	18.0 ± 3.8	23.6 ± 5.5	15.5 ± 2.1	16.9 ± 2.7

Measurements were made at 3 and 10 days following gene transfer in mice that were fasted for 6 h ($n = 6$ for each group). APN: adiponectin (μg/mL). Each value is mean ± S.E.

that adiponectin treatment significantly increased capillary density in the ischemic adductor muscles of WT mice but had no significant effect on capillary density in db1 mice and Lepr$^{db/db}$ mice.

The effects of nonfractionated, recombinant adiponectin at 30 μg/mL on endothelial cell death induced by mitogen deprivation were examined at 5 mmol/L, 10 mmol/L and 30 mmol/L glucose. An MTS-based assay (Figure 4(a)) showed that the adiponectin significantly decreased endothelial cell death at high glucose concentrations (30 mmol/L). Transduction with an adenoviral vector expressing dominant-negative AMPKα2 (Ad-dnAMPK) abrogated the adiponectin-induced decrease in endothelial cell death (Figure 4(b)) [19].

Adiponectin at a concentration of 30 μg/mL increased endothelial cell differentiation into tube-like structures in HUVECs plated on a Matrigel matrix (Figure 5(a)) at physiological glucose concentrations (= 5 mmol/L) consistent with data previously described [12]. But this formation of tube-like structures, seen at 5 mmol/L glucose concentrations, was abrogated despite the administration of adiponectin (= 30 μg/mL) at elevated glucose levels (= 10 mmol/L or

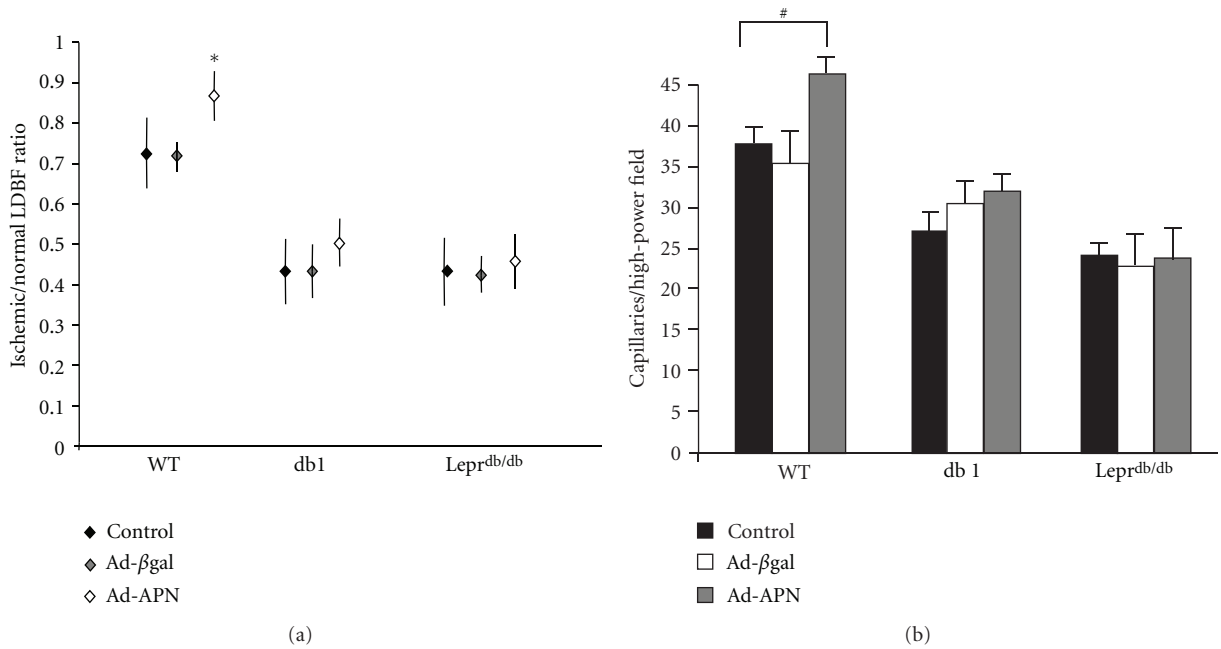

Figure 3: No angiogenic response in ischemic hind limbs of db1 and Lepr$^{db/db}$ mice after adenovirus-mediated gene transfer of adiponectin. (a) Adenoviral vectors expressing adiponectin or β-galactosidase (control) were delivered intravenously via the jugular vein at 3 days prior to surgery. Quantitative analysis of ischemic/nonischemic LDBF ratio in WT, db1, and Lepr$^{db/db}$ mice without adenovirus-mediated gene transfer (= control) or with adenovirus-mediated gene transfer (Ad-βgal and Ad-APN) on postoperative day 14. $^{*}P < 0.01$ versus control of WT, db1, and Lepr$^{db/db}$ mice without adenovirus-mediated gene transfer. (b) Quantitative analysis of capillary density in WT, db1 and Lepr$^{db/db}$ mice in ischemic adductor muscle on postoperative day 14 ($n = 6$) without adenovirus-mediated gene transfer or with adenovirus-mediated gene transfer (Ad-βgal and Ad-APN). Capillary density was expressed as the number of capillaries per high-power field (×400). $^{\#}P < 0.05$.

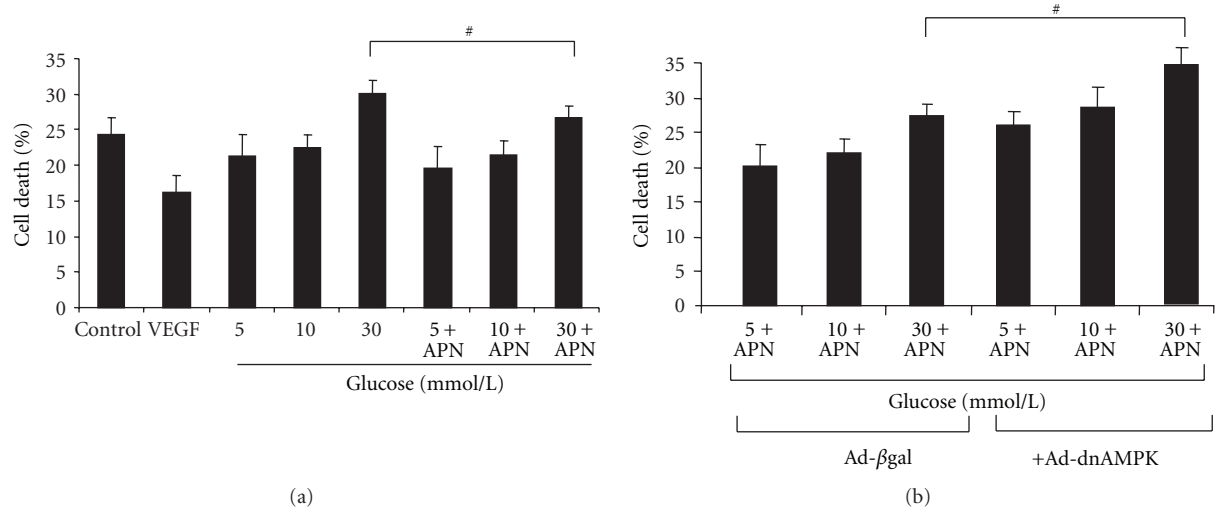

Figure 4: Effects of adiponectin on HUVECs viability. (a) Effect of adiponectin on endothelial cell death induced by serum starvation in control medium (= 0.5% FCS), induced by VEGF (= 10 ng/mL), in 5 mmol/L glucose medium, 10 mmol/L glucose medium, and 30 mmol/L glucose medium without or with adiponectin (= 30 μg/mL) for 48 hours. Cell viability was quantified with an MTS-based assay. VEGF served as additional control. (b) Role of AMPK in the regulation of adiponectin-induced reduction of endothelial cell death in 5 mmol/L glucose medium, 10 mmol/L glucose medium, and 30 mmol/L glucose medium. HUVECs were transduced with an adenoviral vector expressing β-galactosidase (Ad-βgal) (= control) or with an adenoviral vector expressing dominant-negative AMPKα2 (Ad-dnAMPK). After 24-hour infection, cells were treated for 48 hours with adiponectin (= 30 μg/mL) under serum deprivation conditions. Cell viability was quantified with an MTS-based assay.

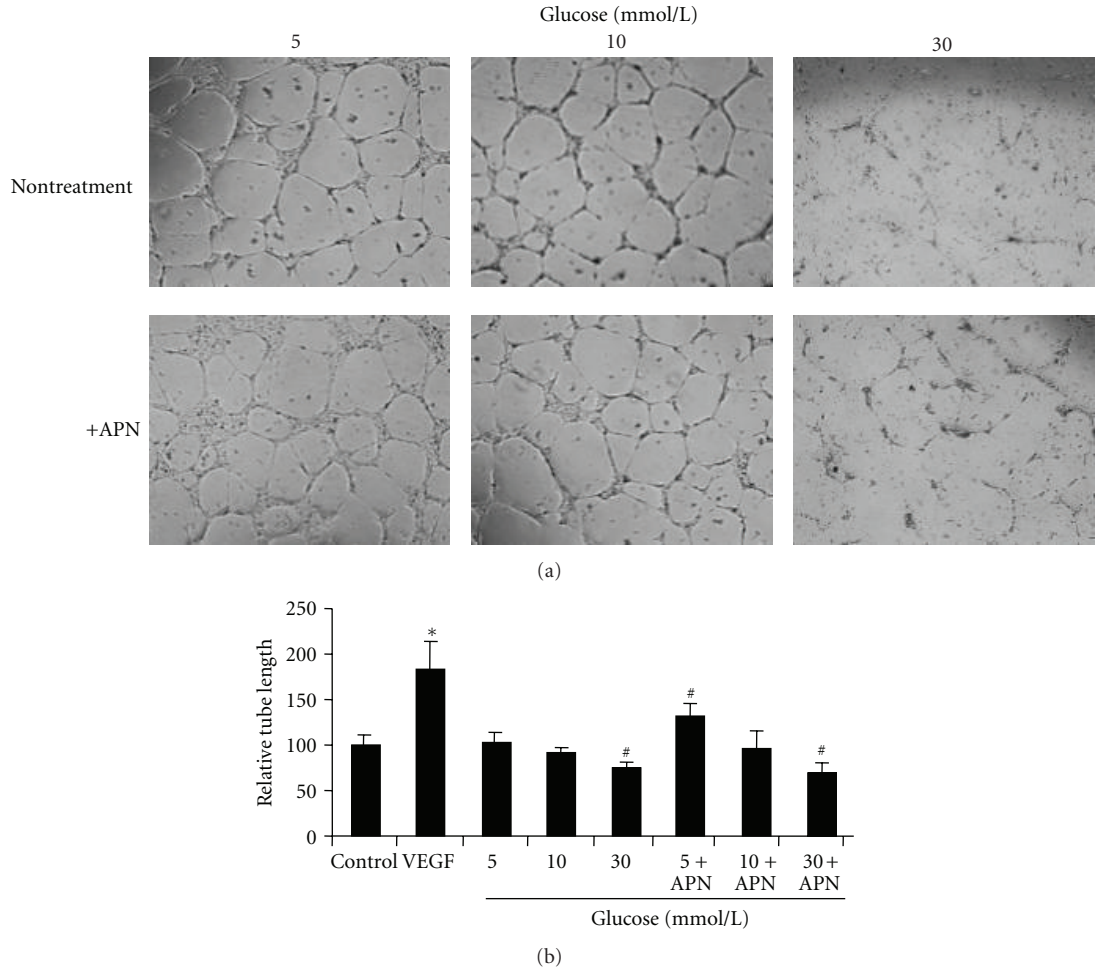

Figure 5: Effects of adiponectin on endothelial cell differentiation into tube-like structures at 5 mmol/L, 10 mmol/L, and 30 mmol/L glucose. (a) Tube formation assays were performed. Representative cultures are shown as indicated. Adiponectin was used at a concentration of 30 μg/mL. (b) HUVECs were seeded on Matrigel-coated culture dishes in control medium (= 0.5% FCS) (= control), induced by VEGF (= 10 ng/mL), in 5 mmol/L glucose medium, 10 mmol/L glucose medium, and 30 mmol/L glucose medium or in 5 mmol/L, 10 mmol/L, and 30 mmol/L glucose in the presence of adiponectin (30 μg/mL). Incubation with VEGF served as additional control. Results are shown as mean ± S.E. Results are expressed relative to control. $^*P < 0.01$, $^\#P < 0.05$ versus control.

30 mmol/L) compared to capillary formation at 10 mmol/L and 30 mmol/L glucose without adiponectin (Figure 5(b)).

4. Discussion

Chronic hyperglycemia leads to microangiopathy, including nephropathy, neuropathy, cardiomyopathy, and peripheral vascular disease [32–35]. A few studies have examined the efficiency of growth factor-mediated angiogenic repair in these diseases. Previously, Rivard et al. or Schiekofer et al. have shown that nonobese diabetic (NOD) mice, a model of Type 1 diabetes or Lepr$^{db/db}$ mice, a mouse model of Type 2 diabetes, suffer from impaired angiogenic repair following hind limb ischemia surgery [22–26, 36–39].

Therefore, the purpose of our study was twofold. First, we compared ischemic-impaired angiogenic repair in streptozocin-treated mice, a model of insulin deficiency, and in Lepr$^{db/db}$ mice, a model of metabolic syndrome and Type 2 diabetes. Second, we evaluated the proangiogenic activity of adiponectin in each of these models. Streptozocin-treated mice or Lepr$^{db/db}$ mice showed decreased reperfusion and capillary density in ischemic hind limbs following femoral artery removal compared to WT mice. The exogenous administration of adiponectin via adenovirus-mediated gene transfer was not able to rescue the impairment in hind limb capillary density in both db1 mice and Lepr$^{db/db}$ mice. In contrast, wild-type mice injected with adiponectin displayed an increase in hind limb reperfusion and an increase in capillary density compared to control mice without injection of adiponectin. This is consistent with previous reports that show that adiponectin promotes angiogenesis due to its ability to stimulate the AMPK-dependent pathways within muscle in WT mice of a C57/BL6 background [18].

Migration of endothelial cells is a key event in angiogenesis that contributes to angiogenic repair in diabetic vasculopathy. Adiponectin stimulates angiogenesis by promoting

cross-talk between AMPK and Akt signaling in human umbilical vein endothelial cells (HUVECs) and promotes angiogenesis due to its ability to stimulate the AMPK-dependent pathways within muscle in WT and adiponectin knockout mice of a C57/BL6 background [12, 13]. In addition, antiapoptotic properties of adiponectin were shown on the vasculature by revealing that recombinant adiponectin dose dependently suppressed apoptosis and caspase-3 activity in HUVECs [14].

Our experiments highlight the importance of the adiponectin AMPK signaling pathway for antiapoptotic properties of adiponectin by showing that the administration of adiponectin significantly decreased endothelial cell death at high glucose concentrations compared to nonadministration of adiponectin. In addition, adiponectin increased endothelial cell differentiation into tube-like structures in HUVECs plated on a Matrigel matrix at physiological glucose concentrations, but this effect was abrogated after the incubation of HUVECs at elevated glucose concentrations. Furthermore, external administration of adiponectin to db1 or $Lepr^{db/db}$ mice that underwent hind limb ischemia did not significantly improve angiogenic repair in comparison to WT mice.

The reasons for the failure of adiponectin to facilitate an increase in capillary density after hind limb ischemia surgery, in db1 or $Lepr^{db/db}$ mice, need further investigation. However, one reason might simply be that the significantly elevated glucose levels in db1 or $Lepr^{db/db}$ mice, compared to WT mice, lead to abrogation of previously reported proangiogenic effects of adiponectin as was shown by our in vitro experiments [12, 13].

We also found that streptozocin-treated db1 or $Lepr^{db/db}$ mice had higher plasma adiponectin concentrations than WT mice according to our baseline data (Table 1). Our results confirm recent reports in an animal model for Type 1 diabetes, which showed that plasma adiponectin levels are higher in Type 1 diabetic patients than in healthy control subjects [40–42]. Follow-up observational studies also showed that higher adiponectin concentrations are associated with an increased risk of incident microvascular complications in Type 1 diabetes [33, 34]. This finding was contrary to those reported for the association between adiponectin and macrovascular complications in Type 2 diabetes, as plasma adiponectin levels have repeatedly been shown to be lower in patients with insulin resistance or macrovascular complications than in control subjects [4, 35]. It is therefore highly likely that adiponectin is regulated differently in Type 1 diabetes than it is in Type 2 diabetes. It remains to be clarified whether elevated levels of adiponectin are pathogenically related to the development of microvascular complications or represent a beneficial counterregulatory response in Type 1 diabetes.

Surprisingly and in contrast to previously reported data, $Lepr^{db/db}$ mice had higher plasma adiponectin concentrations than WT mice according to our baseline data [9, 10]. We surmise that as a consequence of these elevated adiponectin levels, additional administration of adiponectin failed to improve angiogenic repair in this animal model. We speculate that these mice may show elevated adiponectin levels as a result of adiponectin resistance caused by a defect on the adiponectin receptor level that could be linked to the lack of a functional leptin receptor [43, 44]. But this requires further investigation.

Our observations demonstrate that external administration of adiponectin may not be useful in the treatment of angiogenic repair-related complications of Type 1 or Type 2 diabetes with persistent hyperglycemia.

Authors' Contribution

K. Belisle and M. Andrassy and in addition J. Schneider and S. Schiekofer contributed equally to this work.

Conflict of Interests

The authors declared that they have no conflict of interests.

Acknowledgments

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