Published in final edited form as:
*J Pathol*. 2012 June ; 227(2): 165–174. doi:10.1002/path.3960.

# Chronic inhibition of endoplasmic reticulum stress and inflammation prevents ischaemia-induced vascular pathology in type II diabetic mice

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## Abstract

Endoplasmic reticulum (ER) stress and inflammation are important mechanisms that underlie many of the serious consequences of type II diabetes. However, the role of ER stress and inflammation in impaired ischaemia-induced neovascularization in type II diabetes is unknown. We studied ischaemia-induced neovascularization in the hind-limb of 4-week-old db−/db− mice and their controls treated with or without the ER stress inhibitor (tauroursodeoxycholic acid, TUDCA, 150 mg/kg per day) and interleukin-1 receptor antagonist (anakinra, 0.5 μg/mouse per day) for 4 weeks. Blood pressure was similar in all groups of mice. Blood glucose, insulin levels, and body weight were reduced in db−/db− mice treated with TUDCA. Increased cholesterol and reduced adiponectin in db−/db− mice were restored by TUDCA and anakinra treatment. ER stress and inflammation in the ischaemic hind-limb in db−/db− mice were attenuated by TUDCA and anakinra treatment. Ischaemia-induced neovascularization and blood flow recovery were significantly reduced in db−/db− mice compared to control. Interestingly, neovascularization and blood flow recovery were restored in db−/db− mice treated with TUDCA or anakinra compared to non-treated db−/db− mice. TUDCA and anakinra enhanced eNOS-cGMP, VEGFR2, and reduced ERK1/2 MAP-kinase signalling, while endothelial progenitor cell number was similar in all groups of mice. Our findings demonstrate that the inhibition of ER stress and inflammation prevents impaired ischaemia-induced neovascularization in type II diabetic mice. Thus, ER stress and inflammation could be potential targets for a novel therapeutic approach to prevent impaired ischaemia-induced vascular pathology in type II diabetes.



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No conflicts of interest were declared.

**Author contribution statement**

AA, SC, MG, MK, and MP researched data. PK, DH, and MT contributed to discussion and reviewed the manuscript, SB and KM, Project Director, contributed to discussion and wrote the manuscript.

### Keywords

ER stress; inflammation; db/db mice; neovascularization; blood flow

## Introduction

Neovascularization, a process that remodels pre-existing capillaries and forms new vessels, is an essential component of recovery from ischaemic conditions [1]. Thus, the induction of neovascularization is a critical event in the treatment of chronic tissue ischaemia seen in peripheral vascular disease [2]. The induction of neovascularization has been reported to involve a variety of factors that include cell therapy, intermediate signalling, and growth factors [3–5]. Impaired neovascularization is still a major problem in patients with diabetes and cardiac and peripheral vascular diseases [6–10]. The mechanism underlying the defect in neovascularization is still unclear. Thus, a better understanding of the mechanism that enhances neovascularization will greatly facilitate the development of therapeutic strategies to protect tissues from chronic ischaemia.

Recent research has shown that endoplasmic reticulum (ER) stress and inflammation play an important role in the aetiology of type II diabetes [1,11,12]. ER stress causes a complex adaptive signalling response, defined as the unfolded protein response, and is involved in several patho-physiological events. Inflammation is characterized by the presence of a number of inflammatory molecules, including interleukin-1 (IL-1) [13,14]. IL-1 exerts its effect by binding to the IL-1 type 1 receptor. It is well established that high IL-1 levels are correlated with cardiovascular and metabolic diseases [15,16].

The importance of ER stress and inflammation in impaired ischaemia-induced neovascularization, and blood flow recovery in type II diabetes is unknown. It is uncertain whether ER stress and inflammation are related and act together to impair ischaemia-induced neovascularization. Therefore, we tested the hypothesis that the inhibition of ER stress and inflammation could prevent impaired ischaemia-induced neovascularization in a mouse model of type II diabetes by up-regulating the angiogenic factors (NO–cGMP pathway, VEGFR2) and reducing the anti-angiogenic factor (ERK1/2 MAP-kinase).

## Materials and methods

### Animal model and surgery

Four-week-old type 2 diabetic (db−/db−) mice and their wild-type db−/db+ (control) mice (C57BL/6C genetic background) were used in the present study. The reasoning for the use of these mice at such a young age was that at 4 weeks old these mice are not yet diabetic and our goal was to prevent impaired ischaemia-induced neovascularization that occurs in adult db−/db− mice. These studies conformed to the principles of the National Institutes of Health ‘Guide for the Care and Use of Laboratory Animals’ and were approved by the Tulane University Institutional Animal Care and Use Committee. The hind-limb ischaemia procedure was performed in all mice by ligation of the proximal segment of the right femoral artery, as previously described [6,17,18].

### Treatment

Four-week-old db−/db− mice (with normal blood glucose) were treated with the ER stress inhibitor (tauroursodeoxycholic acid, TUDCA, 150 mg/kg per day, daily by intraperitoneal injection) [19,20] or IL-1 receptor antagonist (anakinra, 0.5 μg/kg per day, daily by intraperitoneal injection) just after surgery for 4 weeks.

### Blood glucose

Blood glucose measurements were obtained from tail vein samples using a blood glucose meter (Prestige Smart System HDI; Home Diagnostics, Inc, Fort Lauderdale, FL, USA) once a week in all groups after a 6 h fast [6,21].

### Insulin level

The insulin level was determined at the end of treatment using the Mercodia Ultrasensitive Mouse Insulin ELISA protocol (enzyme immunoassay; Mercodia AB, Uppsala, Sweden), which estimates steady-state insulin resistance [6].

### Cholesterol, adiponectin, and CRP

The plasma cholesterol level was measured using a cholesterol assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), while the plasma levels of CRP and adiponectin were measured using Milliplex™ MAP Luminex MAP™ Technology (Billerica, MA, USA).

### Blood pressure

Systolic blood pressure (SBP) was measured by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan) at the end of the treatment [22]. All mice were trained for tail cuff measurement 1 week before starting the experiments. Measurements were performed at the same time, always at 10 am, with the same number of measurements (ten times) for a single measurement. The temperature at the time of measurement was the same for all groups of mice.

### Laser Doppler measurement of hind-limb blood flow

Each mouse was warmed to a core temperature of 37 °C and then hind-limb blood flow measurements were performed over the region of interest (right and left limbs) before and immediately after surgery, and serially over the 4-week period, with laser Doppler perfusion imaging (LDPI) (Moor Instruments, Axminster, Devon, UK) [6].

### X-ray quantification of hind-limb angiogenesis

Vessel density (all vessels of the ischaemic hind-limb) was assessed by high-definition microangiography at the end of the treatment period, as previously described [6,23]. Data are represented as per cent change, with ischaemic hind-limb values from control mice as 100%.

### Western blot analysis

Western blot analysis of muscle of the hind-limbs was performed as previously described [21,24,25]. The quantification of western blot was determined using Fujifilm-Multi Gauge software. Specific antibodies for total eNOS, phosphorylated eNOS, total VEGFR2, phosphorylated VEGFR2, CD31, and α-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Specific antibodies for total and phosphorylated ERK1/2 MAP-kinase were purchased from Promega (Madison, WI, USA), while phosphorylated C-JNK and MEK were purchased from Cell Signaling Technology.

### Real-time RT-PCR

The mRNA levels of ER stress markers such as C/EBP homologous protein (CHOP) and activating transcription factor 4 (ATF4) were determined in ischaemic hind-limbs from all groups. Total RNA was obtained using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. A total of 1 μg of DNase I-treated RNA reverse was transcribed into cDNA using the High Capacity cDNA

Archive Kit (Applied Biosystems, Foster City, CA, USA) with random hexamers in a 20 μl reaction. PCR was performed in duplicate for each sample using 1 μl of cDNA as a template, 1× TaqMan Universal PCR Master Mix (Applied Biosys-tems), and 10× Taqman Gene Expression Assays (Applied Biosystems) in a 20 μl reaction. Assays-on-Demand (Applied Biosystems) of TaqMan fluorescent real-time PCR primers and probes were used for CHOP (Mm00492097_m1), ATF4 (Mm00515324_m1), and 18S rRNA (Hs99999901_s1), which was used as an endogenous control to normalize the results. Quantitative RT-PCR was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ method. Results are expressed as the relative expression of mRNA in treated mice compared with untreated mice.

### Colorimetric determination of cyclic guanyl-monophosphate (cGMP)

cGMP levels were measured in hind-limb muscles in all groups. We sacrificed mice and immediately harvested and froze the hind-limb muscles in liquid nitrogen. Measurements were performed using a sandwich enzyme-linked immunosorbent assay (ELISA; Cayman Chemical Company) according to the manufacturer's instructions and as previously described [26,27].

### Immunohistochemistry

Immumohistochemistry for endothelial and smooth muscle cells (CD31 and α-actin) and inflammation (macrophage infiltration) was performed as previously described [10,21,25].

### Endothelial progenitor cells (EPCs)

EPCs were isolated from bone marrow as previously described [28]. Mice were sacrificed and bone marrow was harvested into a Petri dish. Bone marrow cells were centrifuged for 15 min at 15 000 rpm at 4 °C. Cells were then re-suspended in red blood cell lysis buffer and centrifuged for 15 min at 15 000 rpm at 4 °C. Cells were then incubated with specific EPC antibodies and the number of EPCs was determined using flow cytometry.

### Statistical analysis

Results are expressed as mean ± SEM. One-way or two-way ANOVA was used to compare each parameter when there were two independent groups. Comparisons between groups were performed using *post-hoc* Bon-ferroni *t*-tests when the ANOVA test was statistically significant. Values of $p < 0.05$ were considered significant. Differences between specified groups were analysed using the Student's *t*-test (two-tailed) for comparing two groups with $p < 0.05$ considered statistically significant.

## Results

### Effect of ER stress and inflammation inhibition on blood pressure, body weight, blood glucose, insulin, cholesterol, and adiponectin levels

At the end of the treatment period, we measured systolic blood pressure, which was similar in all groups of mice (Figure 1A). Body weight and blood glucose levels were measured once a week for 4 weeks. The results revealed that mice treated with TUDCA displayed a significant reduction in body weight and a normalization of blood glucose and insulin levels (Figures 1B–1D), while anakinra (Ana) treatment did not affect blood glucose and insulin levels or body weight (Figures 1B–1D). Blood cholesterol levels were increased, while adiponectin levels were reduced in db−/db− mice compared with control mice (Figures 1E

and 1F). The treatment of db−/db− mice with TUDCA and Ana normalized cholesterol and adiponectin levels compared with control mice (Figures 1E and 1F).

### Effect of ER stress and inflammation inhibition on ER stress markers and macrophage infiltration

Four weeks after femoral artery ligation, the mice were sacrificed and real-time RT-PCR and immunostaining were performed in ischaemic hind-limb muscle from all groups to determine the expression of ER stress markers and macrophage infiltration. Figure 2A shows that *ATF4* and *CHOP* mRNA levels were significantly increased in db−/db− mice compared with control mice. The treatment of db−/db− mice with TUDCA and anakinra significantly reduced *ATF4* and *CHOP* mRNA. Macrophage infiltration was higher in db−/db− mice compared with control and db−/db− mice treated with the ER stress inhibitor and with anakinra (Figure 2B). These results were consistent with the increase in blood CRP levels in db−/db− mice compared with control, which were reduced after TUDCA and Ana treatment (Figure 2C).

### Effect of ER stress and inflammation inhibition on blood flow

After surgery, blood flow was significantly reduced, in all groups of mice, to 5% of the control value. Mice were then treated with TUDCA and anakinra for 4 weeks. Blood flow recovery was measured once a week for a period of 4 weeks in all groups of mice. Our data revealed that blood flow recovery was significantly blunted in the ischaemic hind-limb of db−/db− mice compared with control mice (Figures 3A and 3B). Interestingly, TUDCA and anakinra treatment significantly restored blood flow recovery in db−/db− mice compared with non-treated db−/db− mice (Figures 3A and 3B).

To determine the relationship between ER stress, inflammation, and blood flow recovery in type II diabetes, we performed the following experiments. Control mice were subjected to femoral artery ligation and then injected with tunicamycin (ER stress activator) or IL-1 once a week for 2 weeks. Figure 3C illustrates a significant reduction in blood flow recovery (40%) in control mice with tunicamycin or IL-1 compared with untreated control mice (100%). Importantly, when tunicamycin and IL-1 injections were stopped, blood flow recovery was restored to 100% (Figure 3C).

### Effect of ER stress and inflammation inhibition on vessel density

Vessel density was evaluated by high definition microangiography at the end of the treatment period. After 4 weeks, vessel density was similar between the ischaemic (right) and non-ischaemic (left) hind-limb in control mice (Figures 4A and 4B). However, in db−/db− mice, the ischaemic hind-limb vessel density was significantly reduced compared with the ischaemic hind-limb in control mice. Interestingly, vessel density was significantly increased in the ischaemic hind-limb in db−/db− mice treated with TUDCA and anakinra compared with untreated db−/db− mice (Figures 4A and 4B).

### Effect of ER stress and inflammation inhibition on capillary density

Capillary density, determined by immunostaining with CD31 specific antibody, was significantly reduced in db−/db− mice compared with control mice (Figure 5). The inhibition of ER stress and inflammation induction significantly prevented the decrease in capillary density in db−/db− mice (Figure 5).

### Effect of ER stress and inflammation inhibition on the cGMP, eNOS/VEGFR2/ERK1/2 MAP-kinase pathway

VEGFR2, eNOS phosphorylation, and cGMP levels were significantly reduced, while ERK1/2 MAP-kinase phosphorylation and expression were enhanced in the ischaemic hind-limb of db−/db− mice compared with control (Figures 6A–6D). The inhibition of ER stress and inflammation augmented VEGFR2 and eNOS phosphorylation and cGMP levels, and reduced ERK1/2 phosphorylation and expression in db−/db− mice (Figures 6A–6D). We also demonstrated that C-JNK and MEK phosphorylation was enhanced in db−/db− mice compared with control and db−/db− mice treated with TUDCA and Ana (Figures 6E and 6F). Figures 6G and 6H illustrate a similar number of endothelial progenitor cells from bone marrow in db−/db− and control mice.

## Discussion

The adaptive growth of microvessels is a vital protective mechanism in ischaemia-induced pathology. This adaptive process is impaired in type II diabetes [6,18,29] but the underlying mechanism of impaired ischaemia-induced neovascularization is yet to be determined. We have investigated the therapeutic efficacy of inhibition of endoplasmic reticulum (ER) stress and inflammation on ischaemia-induced neovascularization in a preclinical model of diabetic limb ischaemia.

In the present study, we observed impaired ischaemia-induced neovascularization, ER stress induction, and inflammation subsequent to femoral artery ligation in db−/db− mice. We observed that chronic ER stress inhibition and IL-1 receptor antagonism significantly improved blood flow recovery, as evidenced by enhanced ischaemia-induced neovascularization, vessel and capillary density, and up-regulated angiogenic factors (eNOS–cGMP pathway and VEGFR2 phosphorylation). Endothelial progenitor cells were considered to be important in angiogenesis as previously described [30]. Our results showed that endothelial progenitor cell number was similar in db−/db− and control mice, suggesting that these cells could express a defect in migration, adhesion, and differentiation in db−/db− mice. The improvement in tissue perfusion was consistent with the reduction in MEK, C-JNK, and ERK1/2 MAP-kinase phosphorylation. The inhibition of ER stress and inflammation was assessed by the reduction in ER stress markers (CHOP and ATF4) and the reduction in macrophage infiltration in the ischaemic hind-limb of db−/db− mice, and by the CRP plasma levels.

It is well established that in type II diabetes, the most prevalent metabolic disease worldwide, chronic inflammation contributes to insulin resistance [31]. Interleukin-1 has been reported to play a role in the sustained inflammation observed in type II diabetes [32]. Interestingly, the antagonist of the interleukin-1 receptor (anakinra) was shown to alleviate symptoms in neonatal-onset multisystem inflammatory disease [32]. In our study, systolic arterial blood pressure was similar in all groups, indicating that db−/db− mice are normotensive [21] and that treatment did not affect blood pressure. Interestingly, blood glucose and insulin levels, and body weight were significantly reduced in db−/db− mice treated with TUDCA, but not with anakinra. These data suggest that insulin resistance, hyperglycaemia, and obesity are modulated by the endoplasmic reticulum but not by interleukin-1 expression. In terms of ER stress, our data are in agreement with previous studies showing that inhibition of ER stress reduced the onset of type II diabetes [19,20]. In terms of the effect of the interleukin-1 receptor antagonist, our data are not in agreement with previous studies showing that anakinra treatment improved blood glucose and insulin levels in patients with type II diabetes [32]. The treatment with anakinra did not alter blood glucose and insulin levels or body weight in the mice. This divergence in results could be related to the use of very young db−/db− mice, and it remains to be determined if this

mechanism also exists in older db−/db− mice and in other models of type II diabetes. TUDCA reduced ER stress markers in db−/db− mice and surprisingly, anakinra also reduced ER stress markers. However, further studies are needed to delineate the mechanism that relates inflammation to ER stress.

It is important to mention that there is a difference in effect between TUDCA and anakinra in terms of blood glucose and insulin levels, and body weight regulation. The reductions in ER stress markers and enhanced blood flow recovery were similar in db−/db− mice treated with TUDCA or anakinra. These data suggest that ER stress has an important role in regulating blood glucose and insulin levels, and body weight, while interleukin-1 augmented blood flow recovery is independent of an effect on blood glucose and insulin levels, and body weight. The results showing that cholesterol, adiponectin, and CRP levels were restored after treatment with TUDCA suggested that metabolic function in adipose tissue and the liver is improved.

To refine our insight into the link between ER stress, inflammation, and blood flow recovery in type II diabetes, control mice were subjected to femoral artery ligation and then locally injected with tunicamycin (ER stress activator) or IL-1 once a week for 4 weeks. These results show a significant reduction in blood flow recovery (40%) in control mice with tunicamycin and IL-1 treatment compared with untreated control mice (100%). Importantly, when the tunicamycin and IL-1 treatment was stopped, blood flow recovery was restored to 100%. These data clearly indicate that ER stress and IL-1 are important factors in the impaired ischaemia-induced neovascularization that occurs in type II diabetes. Thus, ER stress and inflammation could be potential targets for therapeutic interventions to reduce ischaemia-induced vascular pathology in type II diabetic patients.

The global prevalence of diabetes is predicted to almost double by the year 2030 from 2.8% to 4.4%. In individuals with diabetes, a significant number of patients will develop lower extremity disease including peripheral neuropathy, foot ulcers, and peripheral arterial disease (PAD), which is a high risk for heart attack and stroke. There is therefore a significant medical need to develop novel therapies to increase the formation of new vessels, especially in patients with type II diabetes. In the present study, we delineated the mechanism responsible for the impaired ischaemia-induced neo-vascularization in type II diabetes, which involves ER stress and inflammation. Our results demonstrated that ER stress and inflammation have differential effects on blood glucose and insulin levels. Although the mechanisms of ER stress and inflammation in type II diabetes are unknown, ER stress and inflammation could be potential targets for therapy to treat vascular occlusive diseases by promoting the formation of new vessels and therefore protecting tissue from ischaemia-induced vascular pathology.

## Acknowledgments

We acknowledge grant support from National Institutes of Health (1R01HL095566; PI: Dr Matrougui; and 5R01HL097111; PI: Dr Trebak).

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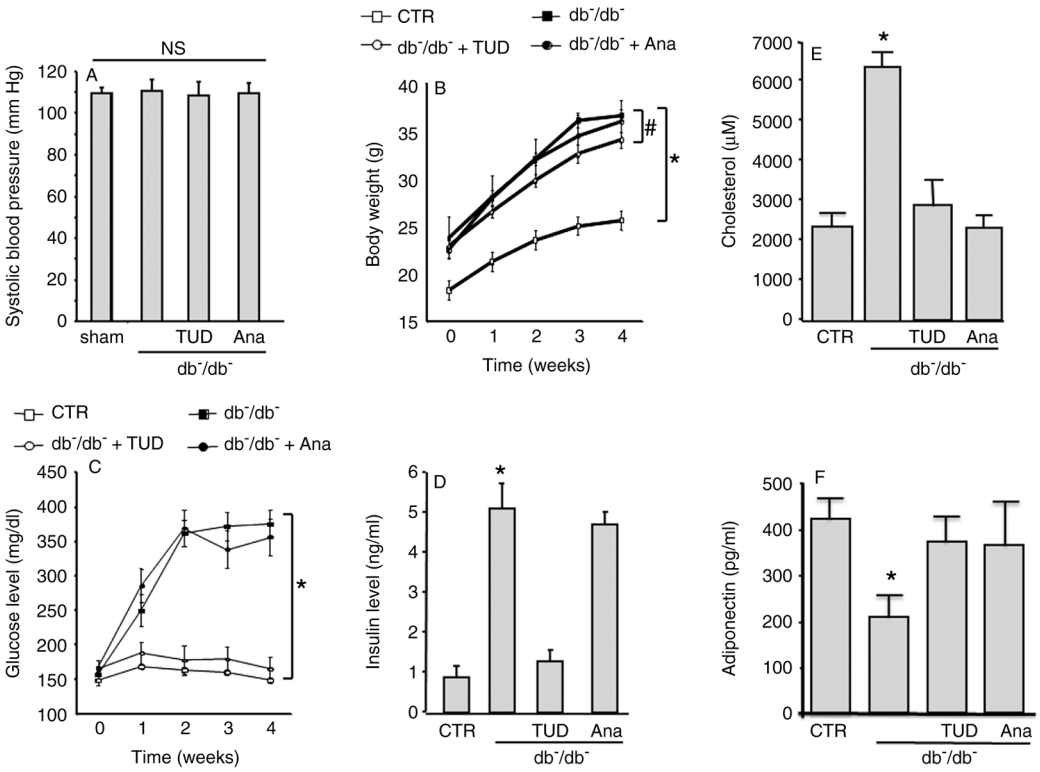


**Figure 1.**

(A) Systolic arterial blood pressure in all groups measured by the tail cuff methods. $n = 7$. (B) Body weight in control (CTR) and db−/db− mice treated with and without TUDCA (TUD) and anakinra (Ana). $n = 7$. $^{*}p < 0.05$ for CTR versus db−/db−; $^{\#}p < 0.05$ for db−/db− versus db−/db− treated with TUDCA. (C) Blood glucose levels in all groups. $n = 7$. $^{*}p < 0.05$ for CTR and db−/db− mice treated with TUDCA versus db−/db− with and without anakinra. (D) Insulin level in all groups. $n = 7$. $^{*}p < 0.05$ for CTR and db−/db− treated with TUDCA versus db−/db− with and without anakinra. (E) Cholesterol levels in all groups. $n = 4$. $^{*}p < 0.05$ for db−/db− versus CTR, db−/db− + TUDCA and Ana. (F) Adiponectin levels in all groups. $n = 4$. $^{*}p < 0.05$ for db−/db− versus CTR, db−/db− + TUDCA and Ana.

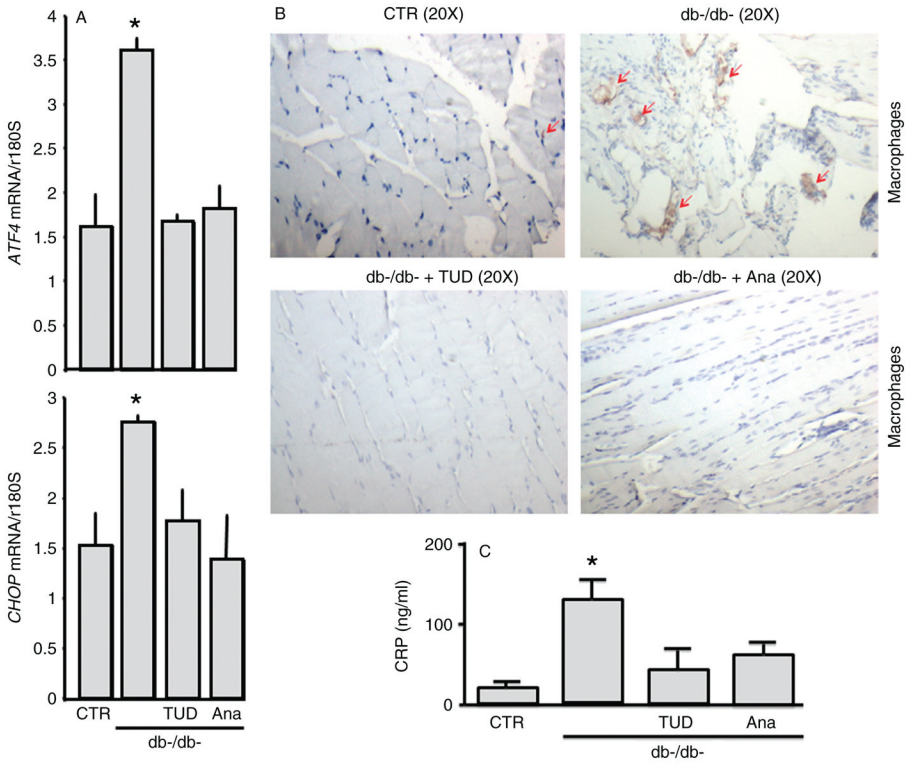


**Figure 2.**

(A) *ATF4* and *CHOP* mRNA levels determined using real-time RT-PCR in ischaemic hind-limb from all groups. $n = 5$. $^{*}p < 0.05$ for db−/db− versus control (CTR) mice and db−/db− mice with and without TUDCA and anakinra (Ana). (B) Immunostaining of macrophages in the ischaemic hind-limb from all groups. (This figure is representative of $n = 5$.) (C) CRP levels in all groups. $n = 4$. $^{*}p < 0.05$ for db−/db− versus CTR, db−/db− + TUDCA and Ana.

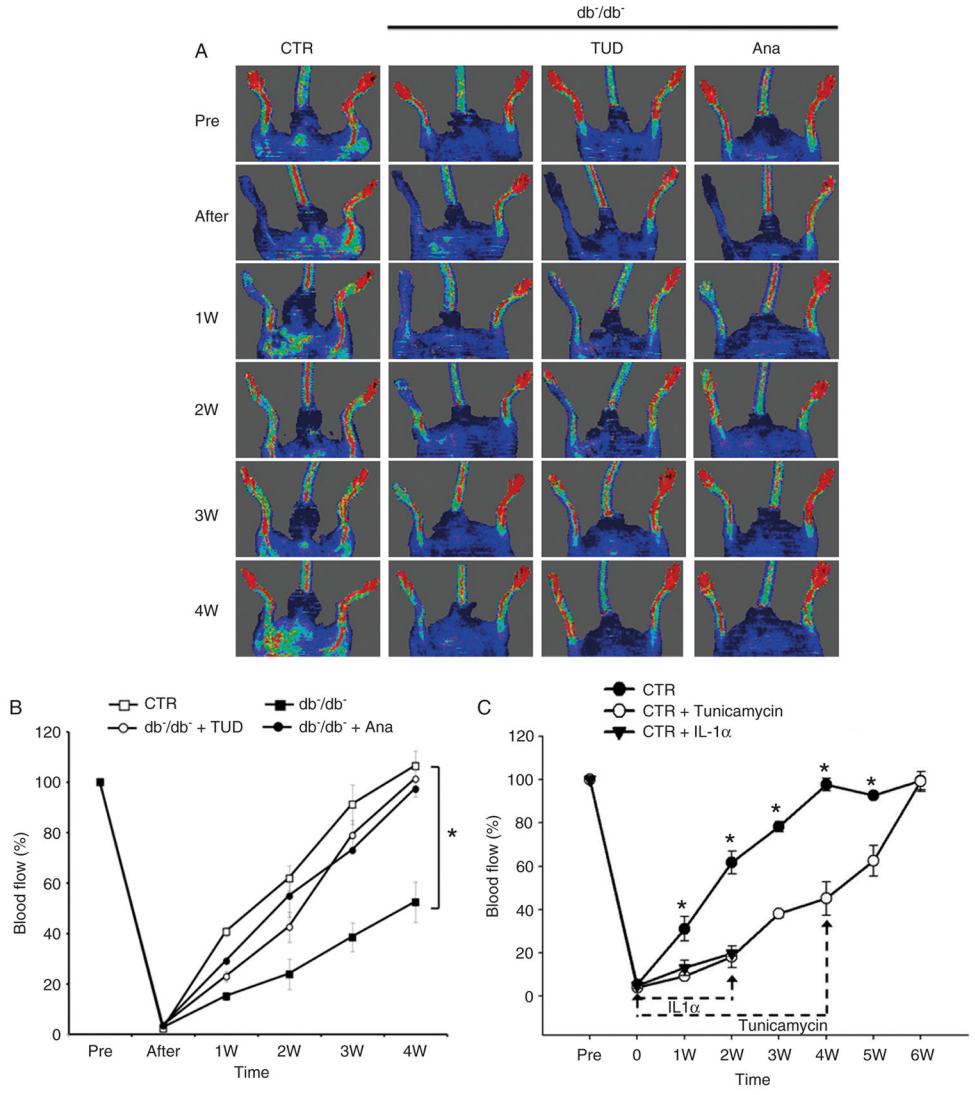


**Figure 3.**

(A) Blood flow recovery measured with a Moor LPDI laser in the ischaemic hind-limb of control (CTR) and db−/db− mice with or without TUDCA and anakinra (Ana) before (Pre) and after surgery, and once a week for 4 weeks. This picture is representative of $n = 8$. (B) Quantitative data for blood flow recovery in all groups of mice. $n = 8$. $^{*}p < 0.05$ for db−/db− mice versus control (CTR) and db−/db− mice + TUDCA and anakinra (Ana). (C) Blood flow recovery measured in ischaemic hind-limb of control (CTR) mice and CTR mice locally injected with tunicamycin (TUN) for 4 weeks or interleukin-1 (IL-1) for 2 weeks. $n = 4$. $^{*}p < 0.05$ for CTR versus CTR + TUN or IL-1.

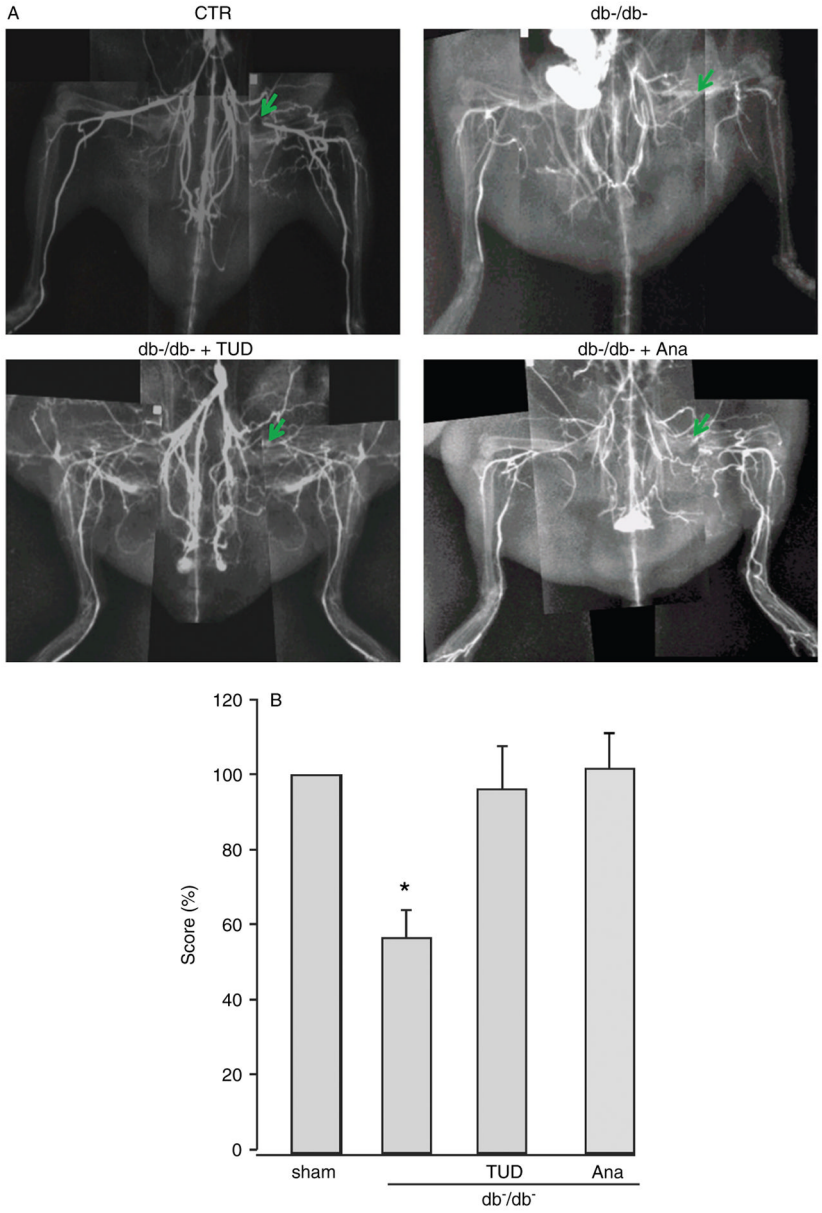


**Figure 4.**

(A) Microangiography performed at the end of the treatment in all groups of mice [control (CTR), and db−/db− with and without TUDCA and anakinra (Ana)]. Contrast medium (barium sulphate, 0.5 μg/ml) was injected into the abdominal aorta and angiography of the right and left hind-limbs was assessed with digital X-ray-acquired images. Images were then assembled to obtain a composite view of the hind-limb. The image is representative of $n = 6$. (B) Quantitative data (score in %) showing ischaemic hind-limb vessel density using Multi Gauge (Fujifilm) by selecting the same area for measurement in all groups. $n = 6$. $^{*}p < 0.05$ for db−/db− mice versus control (CTR) and db−/db− mice + TUDCA and anakinra (Ana).

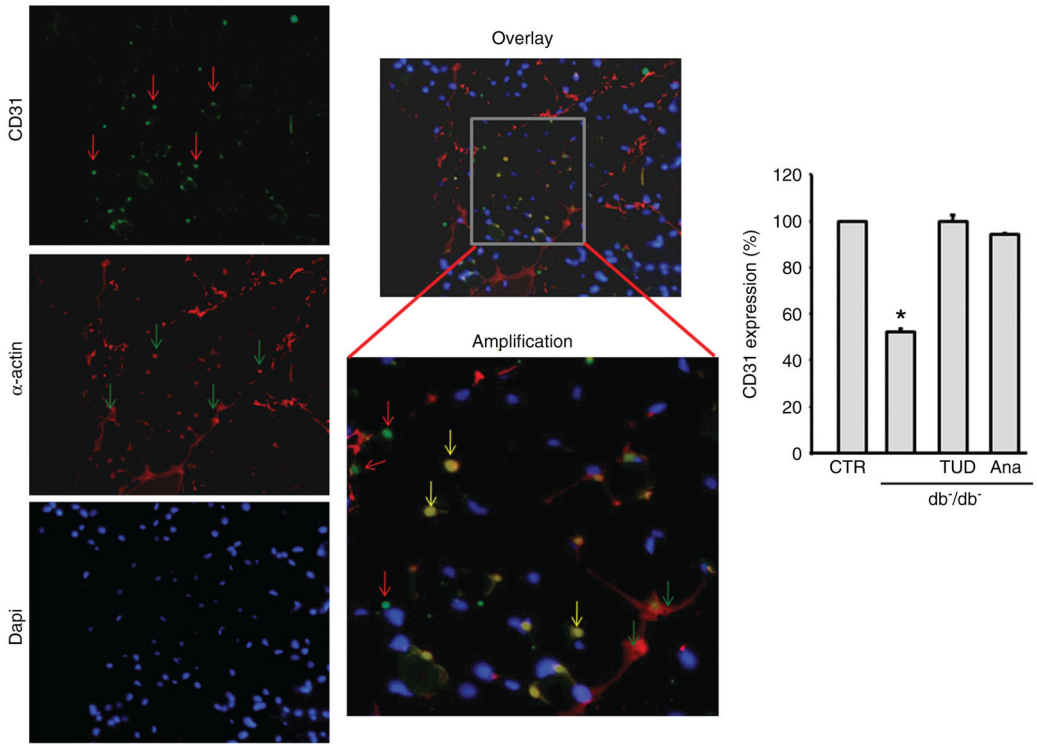


**Figure 5.**
Example of immunostaining with specific antibodies for α-actin (red staining, white arrows) and CD31 (green staining; capillaries). Yellow staining (yellow arrows) represents arterioles which 'merge between green and red'. Quantitative data of CD31 expression in all groups. $n = 5$. $^{*}p < 0.05$ for db−/db− mice versus control (CTR) and db−/db− mice + TUDCA and anakinra (Ana).

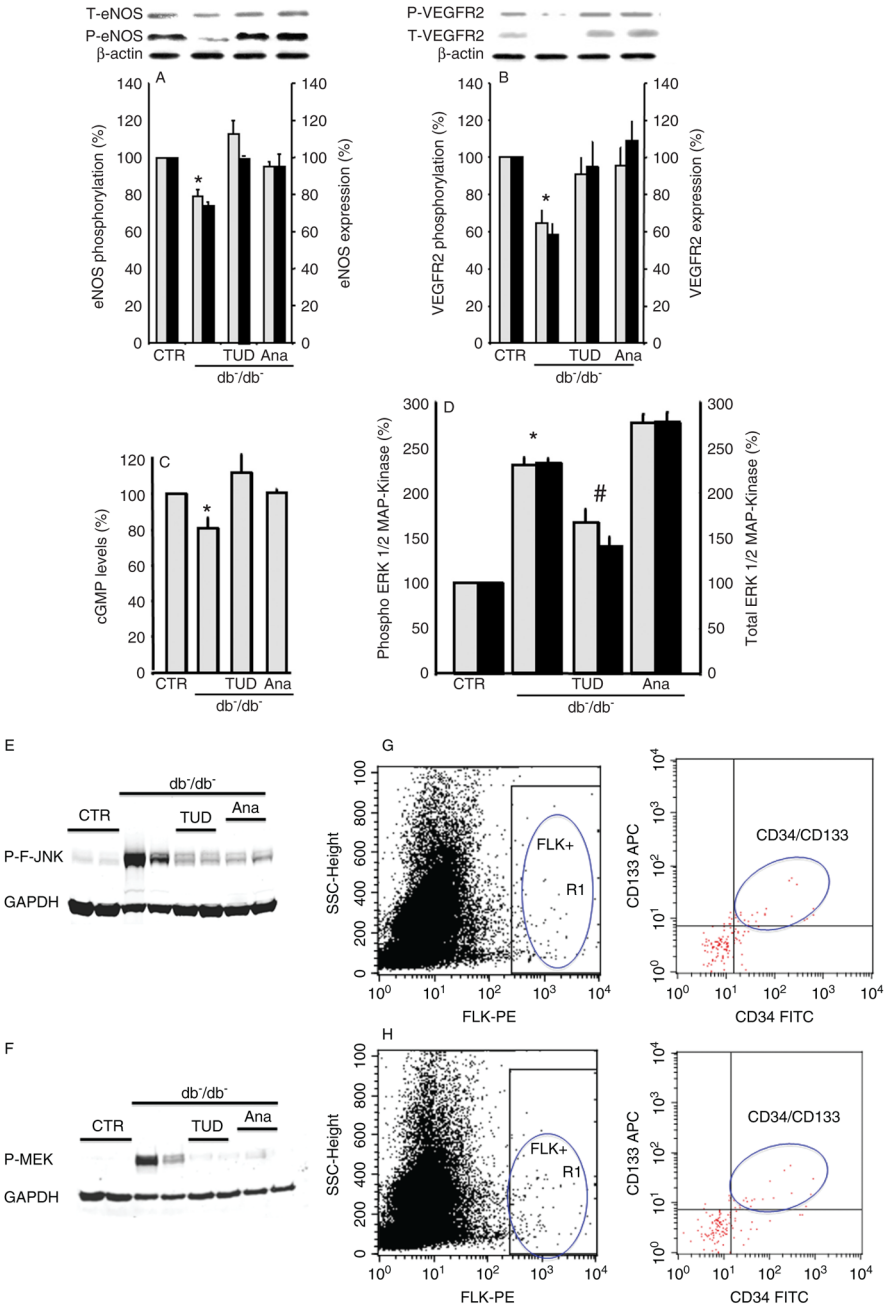


**Figure 6.**

Western blot analysis and quantitative data showing total (T) and phosphorylated (P) eNOS in the ischaemic hind-limb from all groups (A); total (T) and phosphorylated (P) VEGFR2 in ischaemic hind-limb from all groups (B); and total (T) and phosphorylated (P) ERK1/2 MAP-kinase in ischaemic hind-limb from all groups (D). $n = 6$. $^{*}p < 0.05$ for db−/db− mice versus control (CTR) and db−/db− mice + TUDCA and anakinra (Ana). (C) cGMP levels in ischaemic hind-limb from all groups. $n = 6$. $^{*}p < 0.05$ for db−/db− mice versus control (CTR) and db−/db− mice + TUDCA and anakinra (Ana). (E, F) Phosphorylated C-JNK (P-C-JNK) and MEK (P-MEK) in ischaemic hind-limb in all groups of mice. (G, H) Flow cytometry for EPCs in bone marrow from db−/db− and control mice.