

Effects of experimental type 1 diabetes and exercise training on angiogenic gene expression and capillarization in skeletal muscle

Riikka Kivelä,^{*,†,1} Mika Silvennoinen,^{*,†} Anna-Maria Touvra,[†]
T. Maarit Lehti,^{*} Heikki Kainulainen,^{*,†} and Veikko Vihko^{*}

^{*}LIKES Research Center for Sport and Health Sciences, Jyväskylä, Finland; and [†]Neuromuscular Research Center, Department of Biology of Physical Activity, University of Jyväskylä, Jyväskylä, Finland

ABSTRACT Diabetes alters microvascular structure and function and is a major risk factor for cardiovascular diseases. In diabetic skeletal muscle, impaired angiogenesis and reduced VEGF-A expression have been observed, whereas in healthy muscle exercise is known to have opposite effects. We studied the effects of type 1 diabetes and combined exercise training on angiogenic mRNA expression and capillarization in mouse skeletal muscle. Microarray and real-time PCR analyses showed that diabetes altered the expression of several genes involved in angiogenesis. For example, levels of proangiogenic VEGF-A, VEGF-B, neuropilin-1, VEGFR-1, and VEGFR-2 were reduced and the levels of antiangiogenic thrombospondin-1 and retinoblastoma like-2 were increased. Exercise training alleviated some of these changes, but could not completely restore them. VEGF-A protein content was also reduced in diabetic muscles. In line with the reduced levels of VEGF-A and other angiogenic factors, and increased levels of angiogenesis inhibitors, capillary-to-muscle fiber ratio was lower in diabetic mice compared to healthy controls. Exercise training could not restore capillarization in diabetic mice. In conclusion, these data illustrate that type 1 diabetes is associated with reduced skeletal muscle capillarization and the dysregulation of complex angiogenesis pathways.—Kivelä, R., Silvennoinen, M., Touvra, A.-M., Lehti, T. M., Kainulainen, H., Vihko, V. Effects of experimental type 1 diabetes and exercise training on angiogenic gene expression and capillarization in skeletal muscle. *FASEB J.* 20, E921–E930 (2006)

Key Words: angiogenesis · hyperglycemia · growth factor · VEGF

DIABETES IS AN important risk factor for central and peripheral cardiovascular diseases, which increase morbidity and mortality significantly (1–3). It has been shown that diabetes impairs angiogenesis and collateral vessel formation in animal models of ischemia and in ischemic human hearts (4, 5). Hyperglycemia-induced remodeling of skeletal muscle capillary bed is observed, e.g., as shorter capillary diameter and lower capillary-

to-fiber ratio, thus reducing capillary diffusing capacity and disturbing regional hemodynamic regulation (6, 7).

Exercise is recommended in the management of both type 1 and type 2 diabetes. Improved glucose (Glc) uptake and increased insulin sensitivity in skeletal muscle and adipose tissue are the most studied beneficial effects of exercise in diabetes. In healthy individuals, regular endurance exercise training results in the increased capillarization of skeletal muscle (e.g., 8). Studies on capillary density and changes in capillarization after endurance training in diabetic animals and in patients have produced conflicting results (9–12).

Angiogenesis involves multifactorial processes with both proangiogenic and antiangiogenic factors interacting with endothelial cells, smooth muscle cells and the extracellular matrix (ECM). (13). It occurs in a highly regulated manner and varies between tissues and different stimuli. Only a few studies have evaluated the mechanisms by which diabetes affects angiogenesis in skeletal muscle. Most of them have reported reduced expression of VEGF-A, the main angiogenic growth factor, in diabetes (4, 5, 14). However, it has also been suggested that diabetes increases VEGF-A expression in myocardium, but there is resistance to VEGF-A due to the decreased expression of VEGF receptors (15). Recently it was shown that hyperglycemia impaired angiogenesis without altering the expression of vascular growth factors in the chicken chorioallantoic membrane model (16). Acute exercise increases VEGF-A mRNA and protein expression in healthy skeletal muscles (17, 18), but it is not known whether exercise training could modify the diabetes-induced changes.

The importance of studying the effects of diabetes on pro- and antiangiogenic factors in skeletal muscle follows from the well-documented association between diabetes, peripheral vascular complications, and exercise intolerance (19, 20). The purpose of the present study was to investigate changes in angiogenic gene

¹Correspondence: LIKES Research Center, Rautpohjankatu 8a, Jyväskylä FIN-40740, Finland. E-mail: riikka.kivela@likes.fi
doi: 10.1096/fj.05-4780fe

expression and capillarization after experimentally induced type 1 diabetes combined with exercise training in mouse skeletal muscle.

MATERIALS AND METHODS

Animals and experimental setup

Ten- to fifteen-week-old male NMRI mice ($n=60$, Harlan, The Netherlands) with a body wt of 37–43 g were used for the study. The animals were housed in standard conditions (temperature 22°C, humidity 60±10%, light from 8.00 am to 8.00 pm) and had free access to tap water and food pellets (R36, Labfor, Stockholm, Sweden). The experimental procedures were approved by the Animal Care and Use Committee of the University of Jyväskylä, Finland. Mice were randomly assigned into healthy and diabetic groups. The diabetic group received a single peritoneal injection of streptozotocin (STZ, Sigma-Aldrich, France, 180 mg/kg) dissolved in sodium citrate buffer solution (0.1 mol/l, pH 4.5) to induce experimental type 1 diabetes (21). The other group received a sham injection of an equal vol of the buffer. Diabetes was confirmed 72 h after the injection by urine Glc test (Glukotest®, Roche, Germany). Mice were characterized as diabetic when their urine Glc values were greater than 200 mg/dl. The diabetic mice showed symptoms of type 1 diabetes such as polyuria and wt loss. They were not treated with insulin. The mortality of the model was 3% for both healthy and diabetic mice.

Diabetic and healthy animals were randomly assigned into 12 groups ($n=5$ per group), which were either sedentary or trained for one, three or five weeks. Groups were named as follows: sedentary healthy mice (C1, C3, C5), trained healthy mice (T1, T3, T5), sedentary diabetic mice (D1, D3, D5) and trained diabetic mice (DT1, DT3, DT5). Training groups performed 1 h per day of treadmill running at 21 m/min and at an up-hill incline of 2.5° for five days a week. The same person always conducted the training sessions during the dark cycle. Diabetes decreased exercise capacity, but all mice were able to complete the exercise training.

Tissue preparation

Trained mice were killed 24 h after the last training bout together with their sedentary controls. Sample collection was performed 24 h after the last training session to focus on the effects of training and not effects resulting from the last exercise bout. The proximal part of the left quadriceps femoris muscle was mounted in an O.C.T. embedding medium (Miles Laboratories, Elkhart, IN) under a microscope to orientate muscle fibers vertically and snap frozen in isopentane (−160°C) cooled with liquid nitrogen. Calf muscles (soleus, gastrocnemius and plantaris) were snap-frozen and samples were stored at −80°C for further analysis. Serum Glc was analyzed with HemoCue B-Glucose analyzer (HemoCue, Ångelholm, Sweden).

Citrate synthase activity (CS)

The proximal part (~30 mg) of the right calf muscle complex (soleus, gastrocnemius, plantaris) was homogenized in cold 0.2 M NaCl Tris-buffered solution (pH 7.5). The supernatants were used for the assay of citrate synthase activity as described previously (22). Dissolved muscle protein concentration was measured using Bio-Rad Protein Assay according to manufac-

turer's instructions (Bio-Rad, Hercules, CA). Enzyme activities were expressed as units per mg of dissolved protein.

Immunohistochemistry

Quadriceps femoris muscles from mice trained for five weeks and their respective controls were used for immunohistochemical analyses. Adjacent transverse cross-sections (10- μ m thick) were cut with a cryomicrotome. The primary antibodies used were mouse anti-human dystrophin (DYS2, Novocastria, Newcastle on Tyne, UK), rat antimouse CD31/PECAM-1 (BD Biosciences Pharmingen, San Diego, CA), goat antimouse VEGF-A (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-mouse VEGF-B (Santa Cruz Biotechnology) and goat anti-mouse VEGFR-2 (R&D Systems, Minneapolis, MN). Peroxidase staining was performed with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and DAB or AEC (Sigma, Saint Louis, MO) was used as a chromogen. Stained sections were studied under a light microscope (Olympus BX-50, Olympus Optical, Tokyo, Japan). TEMA image analyzing software (TEMA Image-Analysis System, Scan Beam, Denmark) was used to analyze the images. Cross-sectional area of muscle fibers (CSA), capillary density (number of capillaries per mm²) and capillary-to-fiber ratio were calculated. The number of muscle fibers analyzed per sample ranged from 300 to 500.

VEGF-A ELISA

A portion of the right calf muscle complex was homogenized in RIPA buffer (PBS, 1% Igepal, 0.5% Na-deoxycholate, 0.1% SDS with protease inhibitors PMSF, aprotinin and leupeptin). VEGF-A was determined by a commercial high-sensitivity ELISA kit according to the manufacturer's instructions (R&D Systems). VEGF-A levels were obtained by use of a microplate reader at 450 nM and corrected by readings at 540 nM. VEGF-A concentration was related to the total protein content of the homogenate. Dissolved muscle protein concentration was measured using the Bio-Rad Protein Assay according to the manufacturer's instructions (Bio-Rad). Measurements were done in duplicate.

RNA extraction

Total RNA was isolated from the left calf muscle complex with Trizol Reagent (Invitrogen, Carlsbad, CA) and further purified with RNeasy kit (Qiagen, Valencia, CA) according to the manufacturers' protocols. Concentration and purity of RNA was determined spectrophotometrically at wavelengths 260 and 280 nM. Integrity was checked with agarose gel electrophoresis. For microarray analysis, RNA samples were pooled within each group resulting in 12 arrays. Individual RNA samples were used for real-time PCR.

Oligonucleotide array analysis

Oligonucleotide array analyses for pooled RNA samples were performed using the Affymetrix Gene Chip MG U74Av2 (Affymetrix, Inc., Santa Clara, CA), which represents 6000 known genes and 6000 ESTs. The Finnish DNA Microarray Centre at the Turku Centre for Biotechnology conducted the microarray analyses according to the instructions supplied by Affymetrix. Arrays were scanned using a GeneArray Scanner G2500A (Agilent, Palo Alto, CA) and images were analyzed with Microarray Suite 5.0 software (Affymetrix). All chips were scaled (global scaling) to the target intensity of 50 to minimize differences between chips caused by physical differ-

ences in chips, hybridization efficiencies, and manual laboratory work. The data were subjected to robust normalization to reduce errors caused by binding capacity and linearity differences between probe sets. All samples were quality-checked according to the recommendations of Affymetrix before comparison analysis to determine differentially expressed genes. The following comparisons were performed using Microarray Suite 5.0: trained (T) vs. control (C), diabetic (D) vs. control, trained diabetic (DT) vs. control and trained diabetic vs. diabetic. The comparisons were made at each time point. Transcripts had to meet multiple criteria before they were regarded as differentially expressed. Changes in expression had to be significant (increased or decreased) according to Microarray Suite 5.0 algorithms and the two-based log ratio of expressions > 0.3 or < -0.3 . Transcripts had to be at least marginally present in one of the two compared samples, and they also had to be present at least in three samples. GeneSpring 6.1 (Silicon Genetics, Redwood City, CA) software was used in applying the last mentioned filter, when drawing up gene lists and preliminary result tables. The complete data set is publicly available in the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>; accession number GSE1659).

Real-time quantitative PCR

The ABI Prism 7700 Sequence Detection System was used to perform TaqMan probe-based real-time PCR reactions (Applied Biosystems). RNA was reverse-transcribed to cDNA with a High-Capacity cDNA Archive Kit (Applied Biosystems). Primer and probe sets were designed and synthesized by Applied Biosystems (GAPDH: Mm99999915 m1, VEGF-A: Mm00437304 m1, Thrombospondin-1: Mm00449022 m1, CGTF: Mm00515790 g1). Primer pairs were designed so that they overlapped an exon-exon boundary to avoid interference from possible genomic DNA contamination. Target genes in the sample were quantified according to the corresponding gene-specific standard curve. As an endogenous control to correct for potential variation in RNA loading or efficiency of the amplification, GAPDH mRNA was used. In the microarray data, GAPDH showed the steadiest expression in all conditions when the normally used housekeeping genes were compared. GAPDH is also considered the most stable internal control in exercise studies (23). All samples were analyzed in triplicate.

Statistical analysis

Student's *t* test was used in determining statistically significant differences between healthy and diabetic or sedentary and trained animals in body wt, serum Glc concentration and muscle citrate synthase activity. Depending on the normal distribution of the data, one-way ANOVA or nonparametric Kruskal-Wallis with Mann-Whitney *U*-tests were used to analyze differences in mRNA and protein levels, capillarization, and fiber CSA. For oligonucleotide array, the one-sided Wilcoxon's signed rank test (WSR) was applied to the perfect

match (PM) and mismatch (MM) intensities of each probe set to determine which genes were expressed above the background. This nonparametric test is robust, insensitive to outliers, and does not assume a normal data distribution (24). Genes were called as significantly expressed at $P \leq 0.04$. Statistical algorithms based on the WSR test were also used to determine significant differential expression in the comparative analyses between treatment groups. Gene expression was considered significantly increased at $P \leq 0.0025$ and decreased at $P \geq 0.9975$. Calculation of the magnitude of the change in expression was based on differences between the intensities of the corresponding probe pairs (PM-MM) across the two arrays and one-step Tukey's biweight estimate statistics. Aforementioned nonparametric test is suitable for expression analysis due to its insensitivity to the intensities of the outlier probe pairs (25).

RESULTS

Body wt, blood Glc, and citrate synthase activity

Effects of diabetes alone or combined with endurance training on selected physiological parameters are shown in **Table 1**. The body weights of the mice in groups D and DT decreased during the experiment ($P < 0.001$). Serum Glc concentrations in trained and untrained diabetic mice were five times higher than in healthy control mice ($P < 0.001$). Serum Glc tended to be lower in trained diabetic than in untrained diabetic mice ($P = 0.07$). Citrate synthase activity decreased in the skeletal muscles of diabetic mice ($P < 0.05$). Trained healthy and trained diabetic mice both had higher citrate synthase activity than their respective untrained controls ($P < 0.05$).

Expression changes in angiogenesis-related genes

Streptozotocin-induced diabetes affected the expression of several genes involved in angiogenesis. Genes of interest were selected on the basis of the literature and Affymetrix gene annotations. Diabetes decreased the mRNA levels of the major angiogenic growth factor VEGF-A as well as the levels of VEGF-B, neuropilin-1, VEGF receptor-1 (Flt-1) and VEGF receptor-2 (Flk-1). mRNA levels of myoglobin, MnSOD (SOD2), integrin alpha V, and secreted acidic cysteine-rich glycoprotein (Sparc) were also down-regulated. In contrast, the amount of thrombospondin-1 (TSP-1) mRNA, an inhibitor of angiogenesis, was significantly increased in diabetic muscles as well as the levels of retinoblastoma-like-2 (Rbl-2), connective tissue growth factor (CTGF),

TABLE 1. Effects of diabetes and endurance training on body weight (BW), serum glucose, and citrate synthase (CS) activity

	Healthy control	Healthy trained	Diabetic control	Diabetic trained
Change in BW (%)	0.1 ± 5.1	2.0 ± 5.5	-23.4 ± 10.2***	-19.7 ± 10.6***
Serum glucose (mmol/l)	10.3 ± 1.5	9.9 ± 1.3	54.9 ± 5.7***	50.5 ± 6.8***
CS Activity (nmol·min ⁻¹ ·mg ⁻¹)	469 ± 99	553 ± 140*	400 ± 74*	501 ± 60†††

Each group consists of mice from all three time points ($n = 15$ in each group). Values are presented as mean ± SD. * $P < 0.05$ vs. healthy control; *** $P < 0.001$ vs. healthy control; ††† $P < 0.001$ vs. diabetic control.

TABLE 2. Significant changes in mRNA levels of angiogenesis-related genes.

Accession	Gene name	Gene/Protein	D1	D3	D5	DT1	DT3	DT5	T1	T3	T5
U43836	Vegfb	Vascular endothelial growth factor B	0.87	0.71	0.54	1.15	0.87	0.81	1.32	1.23	1.23
M95200	Vegfa	Vascular endothelial growth factor A	0.62	0.62	0.44	0.76	0.76	0.66	0.93	1.15	1.07
D50086	Nrp	Neuropilin	0.66	0.54	0.57	0.81	0.57	0.87	1.07	1.00	1.07
L35528	Sod2	Superoxide dismutase 2, mitochondrial	0.57	0.62	0.66	0.81	0.81	0.76	1.07	1.15	1.15
X04405	Mb	Myoglobin	0.35	0.50	0.62	0.71	0.66	0.76	1.07	1.15	1.15
M15832	Col4a1	Procollagen, type IV, alpha 1	0.47	0.54	0.66	0.57	0.54	0.76	0.66	0.93	1.00
X04647	Col4a2	Procollagen, type IV, alpha 2	0.57	0.76	0.76	0.66	0.76	0.87	0.81	1.15	1.07
AI840158	Angptl2	Angiopoietin-like 2	0.44	0.76	0.62	0.62	0.81	0.76	0.81	1.00	1.00
AI843901	Itgav	Integrin alpha V	0.62	0.76	0.93	0.66	0.71	0.76	0.76	0.81	0.93
Z50013	Hras1	Harvey rat sarcoma virus oncogene 1	0.57	0.66	0.76	0.81	0.71	0.76	0.87	0.93	0.81
X04017	Sparc	Secreted acidic cysteine rich glycoprotein	0.47	0.57	0.81	0.57	0.71	0.81	0.76	1.07	1.15
X70842	VEGFR-2/Flk1	Kinase domain insert protein receptor	0.47	0.71	0.71	0.62	0.71	0.93	0.66	1.15	1.15
D88689	VEGFR-1/Flt1	FMS-like tyrosine kinase 1	0.93	0.71	0.81	1.00	0.87	1.15	1.15	1.07	1.32
U88327	Socs2	Suppressor of cytokine signaling 2	0.93	1.07	1.15	0.87	0.62	0.81	0.87	0.81	0.87
AF003695	Hif1a	Hypoxia inducible factor 1, alpha	0.93	1.23	1.23	0.81	0.93	1.00	0.81	0.81	0.87
M70642	Ctgf	Connective tissue growth factor	1.41	1.41	1.52	0.93	0.93	1.87	0.93	0.66	0.87
M32490	Cyr61	Cysteine rich protein 61	1.87	2.46	1.52	1.87	1.52	1.23	1.41	0.93	0.66
D88791	Csrp3	Cysteine and glycine-rich protein 3	3.73	2.83	2.14	1.87	1.00	2.46	0.93	1.00	1.15
V00835	Mt1	Metallothionein 1	11.31	8.00	2.30	6.06	3.25	7.46	3.48	1.41	0.87
K02236	Mt2	Metallothionein 2	10.56	8.00	4.00	7.46	4.00	8.57	4.93	1.23	1.00
AF064088	Tieg1	TGFβ inducible early growth response 1	2.83	1.52	1.74	1.74	1.32	1.15	1.23	0.87	0.54
AW049795	Tbrg1	TGF beta regulated gene 1	2.14	1.52	1.32	1.23	1.32	1.32	1.07	0.93	0.93
AW120719	Eif2b1	Eukaryotic translation initiation factor 2B	1.87	1.32	1.23	1.15	1.32	1.32	1.07	0.93	1.00
M62470	Thbs1	Thrombospondin 1	1.74	2.14	2.83	1.63	1.87	2.64	0.71	0.81	0.66
U36799	Rbl2	Retinoblastoma-like 2	1.23	1.32	1.41	1.32	1.32	1.52	1.07	0.87	1.00
AI849838	Cull1	Cullin 1	1.74	1.23	1.32	1.23	1.23	1.32	1.23	1.00	1.00
U42384	Fin15	Fibroblast growth factor inducible 15	1.52	1.63	1.23	1.23	1.41	1.07	1.07	1.41	1.15
AI843709	Il6st	Interleukin 6 signal transducer	1.63	1.32	1.41	1.41	1.23	1.23	1.15	0.93	0.87
AF061503	Bop1	Block of proliferation 1	1.63	1.87	0.93	1.63	1.74	1.52	1.23	1.52	1.32

Gene expressions are expressed in relation to the control group at the same time point. Statistically significant changes are color-coded: red = up-regulated (two-based log of ratio (sample/control) ≥ 0.3 and $P \leq 0.0025$), green = down-regulated (two-based log of ratio (sample/control) ≤ -0.3 and $P \geq 0.9975$), yellow = diabetes-induced change in expression was attenuated by exercise (significant change in expression in opposite direction in comparisons DT vs. D and D vs. C). Group abbreviations: D = diabetic, DT = diabetic trained, and T = healthy trained, numbers = time of the treatment in weeks.

Hif-1 α and cysteine-rich protein 61 (Cyr61). Also the levels of metallothionein 1 and 2 were markedly increased in diabetic muscles. Endurance training alleviated the diabetes-induced changes in the mRNA levels of some of the genes (e.g., VEGF-A, VEGF-B, neuropilin-1, myoglobin, Hif-1 α , and metallothioneins), but did not significantly decrease the levels of TSP-1 or Rbl-2. Significant changes in the angiogenesis-related genes are presented in the **Table 2**. The expression of VEGF-A, TSP-1, and CTGF was also confirmed with real-time RT-PCR. The results were well in line with those obtained from the microarray and are presented in **Fig. 1**.

VEGF-A protein

VEGF-A protein concentration in skeletal muscle homogenates was reduced in sedentary diabetic mice after three and five weeks of diabetes compared with healthy controls ($P < 0.05$) (**Fig. 2**). In the endurance-trained diabetic groups VEGF-A protein content was significantly decreased after only 5 wk. After 3 wk, trained diabetic animals tended to have higher VEGF-A content than sedentary diabetic mice ($P = 0.086$). There was no significant change in VEGF-A content

in healthy trained mice compared to the healthy sedentary group.

Localization of VEGF-A, VEGF-B, and VEGFR-2

In **Fig. 3** the representative images of the localization of VEGF-A, VEGF-B, and VEGFR-2 proteins are presented. VEGF-A protein was localized under sarcolemma in some of the muscle fibers, in the endothelium of larger blood vessels and in some capillaries. VEGF-B was stained strongly in the larger blood vessels, in certain interstitial cells and in only a few muscle fibers. Weak staining was also observed in some capillaries. VEGFR-2 protein was expressed in most of the capillaries and in vessels without thick smooth muscle cell layer situated close to arteries and large veins, which are probably lymphatic vessels. No differences were observed between the healthy and diabetic mice in the localization of these proteins.

Capillarization and fiber cross-sectional area

Both sedentary and trained diabetic mice showed a significant decrease in muscle fiber cross-sectional area

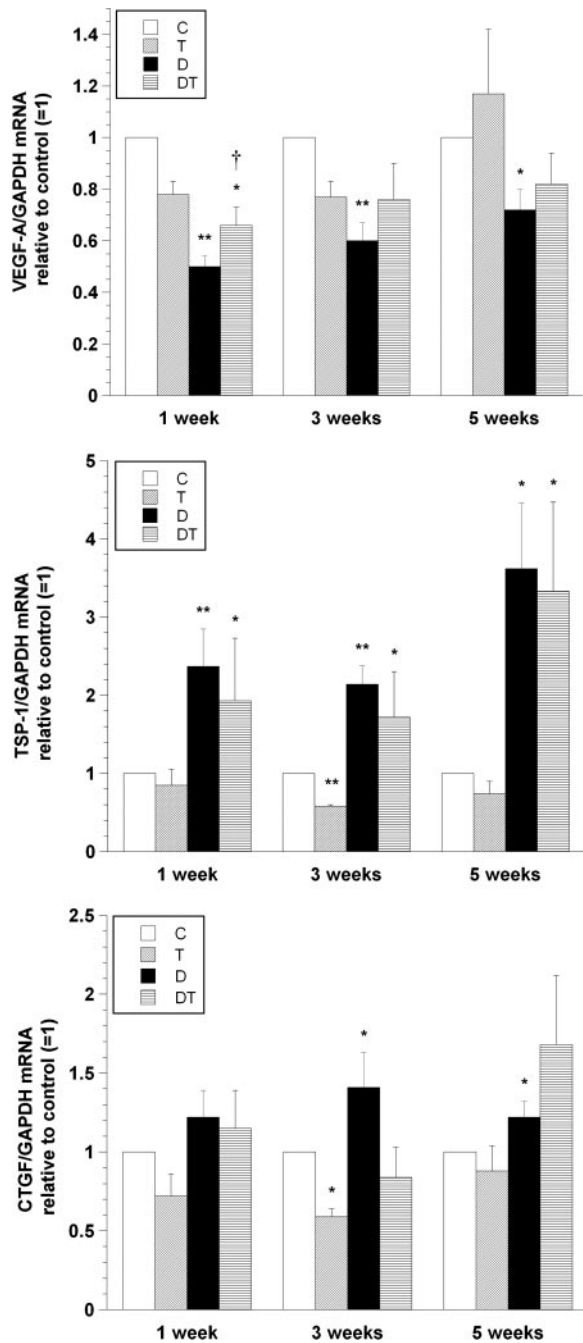


Figure 1. Expression of VEGF-A was decreased in skeletal muscle of diabetic sedentary mice, and exercise training could significantly reverse this after the first week. In contrast, thrombospondin-1 (TSP-1), and connective tissue growth factor (CTGF) mRNA expression were increased in diabetic muscles. Expression changes in trained healthy (diagonally striped bars), sedentary diabetic (black bars) and trained diabetic (horizontally striped bars) groups were compared to sedentary healthy controls (white bars, set to value 1). Values are mean \pm SE. * $P < 0.05$ vs. healthy sedentary controls, $^{\dagger}P < 0.05$ vs. respective sedentary diabetic (=effect of training). Note the varying scale on the y-axis due to the magnitude and direction of the changes.

(CSA) after 5 wk compared to healthy controls ($P < 0.05$) (Fig. 4A and Fig. 5C, D). Capillary density ($\text{cap} \cdot \text{mm}^{-2}$) was not significantly changed due to diabetes, although it was slightly increased due to the reduced fiber cross-sectional area (Fig. 4C). Instead, the capillary-to-fiber ratio, which is commonly used to describe capillary supply to muscle fibers, was decreased in both diabetic groups compared to the healthy control group ($P < 0.05$), but was not different between the trained and sedentary diabetic mice (Fig. 4B). Healthy trained mice tended to have greater capillary-to-fiber ratio than control mice, but the difference was not statistically significant.

DISCUSSION

Angiogenesis is modulated by the balance between the influence of positive and negative stimuli on the growth of capillaries. In the present study, we determined separate and combined effects of diabetes and exercise training on the expression of pro- and antiangiogenesis genes and proteins and on the capillarization in mouse skeletal muscle. The high blood Glc concentration and considerable loss of body wt confirmed that streptozotocin had induced diabetes in the studied mice. The significant atrophy of the muscle fibers seen in this study is also normal in diabetic animals, if they are not treated with insulin.

Diabetes decreased the mRNA concentration of many genes known to be involved in the regulation of angiogenesis, most interestingly those of VEGF-A and VEGF-B together with their receptors VEGFR-1/Flt1, VEGFR-2/Flk1, and neuropilin-1. The effect of diabetes on the VEGF-B and VEGF receptor mRNAs in skeletal muscle has not been reported earlier. In diabetic rabbits a reduction in VEGF-A mRNA levels in ischemic skeletal muscle has been observed previously (4). Decreased cardiac expression of VEGF-A and its receptors VEGFR-1 and -2 has been found in diabetic rats and in the human myocardium from diabetic patients (14). Treating the rats with insulin normalized these changes. Similarly, in cell cultures high Glc levels have been reported to inhibit VEGF-A production (26) and insulin, in turn, to enhance it (27).

In addition to the mRNAs, we studied the localization of three important angiogenesis proteins, VEGF-A, VEGFR-2, and VEGF-B, in healthy and diabetic muscles. VEGF-A localized to myofibers and endothelial cells as has been shown earlier (e.g., in 28) in skeletal muscles. In line with previous studies we found VEGFR-2 in endothelial cells (28). In ischemic muscle VEGFR-2 is also localized in myofibers (28), but in our study VEGFR-2 was not found in muscle fibers in either healthy or diabetic muscles. To our knowledge, the localization of VEGF-B protein in skeletal muscle has not been reported earlier. The present findings showed that VEGF-B is localized mainly in larger blood vessels, capillaries, and interstitial cells. Similarly to VEGF-A, it was also detectable under the sarcolemma in a few

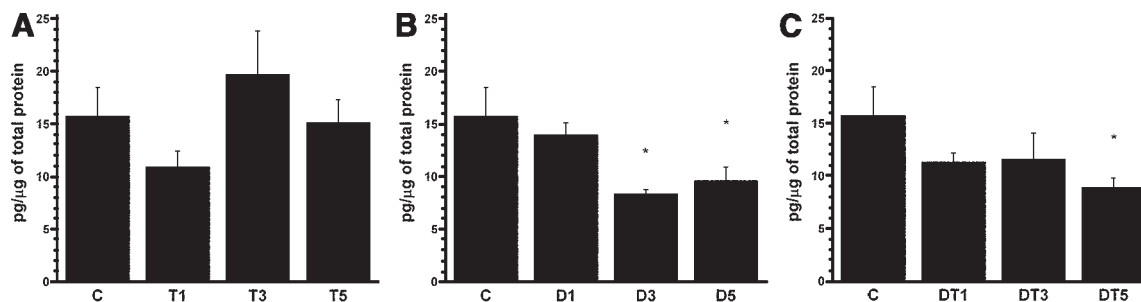


Figure 2. Training did not change skeletal muscle VEGF-A protein content significantly in healthy mice (A). In sedentary diabetic mice, VEGF-A content was decreased after 3 and 5 wk (B). In trained diabetic mice, the decrease occurred after 5 wk (C). Values are mean \pm SE. * $P < 0.05$ vs. healthy sedentary controls.

muscle fibers. The exact role of VEGF-B in angiogenesis is not clear at the moment, but it is abundantly produced both in myocardium and skeletal muscle (29). The present results confirm the presence of both VEGF-B mRNA and protein in skeletal muscle and its localization to blood vessels support its role in the maintenance of blood vessel endothelium. The localization of these three proteins was similar in healthy and diabetic skeletal muscle.

Diabetes significantly reduced also the amount of myoglobin and superoxide dismutase (SOD) 2 (MnSOD/SOD2) mRNAs. VEGF-A has been shown to enhance myoglobin expression in skeletal muscle both in vivo and in vitro. This may be a mechanism, which improves tissue oxygenation without neovascularization (30). In our study, the amount of both VEGF-A and myoglobin mRNA was reduced in diabetic mice, possibly influencing oxygen transport to muscles. In addition to being a strong angiogenic factor, VEGF-A seems to have a role in coordinating the growth and phenotype of myofibers and their interaction with capillaries (30, 31), which may become compromised in diabetic muscles when VEGF-A levels are decreased. Reactive oxygen species (ROS) are increased in hyperglycemic tissues (32), thus reduced MnSOD in diabetic muscle may further predispose myofibers and endothelial cells to oxidative damage.

Diabetes also increased mRNA levels of several genes involved in angiogenesis. The most significant of these were the increases in TSP-1 and Rbl-2, both of which are known to inhibit angiogenesis (33, 34). Increased expression of TSP-1 in the vessel wall of diabetic Zucker rats has been reported earlier (35), and it was proposed, that it could be a direct response of vascular cells to Glc and, thus a link between diabetes and atherosclerotic complications. The present results extend this finding from aorta to peripheral muscle tissues. Overexpression of retinoblastoma-like 2 (Rb2/Rbl2) decreased VEGF-A mRNA and protein levels in two tumor cell types together with inhibited tumor angiogenesis, which suggest that Rbl2 is a potent VEGF-A inhibitor (34). In our experiment, diabetic muscles expressed higher mRNA levels of retinoblastoma-like 2 and lower mRNA levels of VEGF-A than healthy muscles. Diabetes also affected the expression of another matricellular

protein Sparc, which interacts with VEGF-A and inhibits spreading of endothelial cells (36).

Stress-inducible metallothionein-1 and -2 mRNA levels were over 10-fold higher after the first week of diabetes. Thereafter, the levels slightly attenuated, but remained still elevated, throughout the whole experiment. Hyperglycemia-induced overproduction of ROS may induce the observed increase in metallothionein-1 and -2, which can alleviate symptoms of oxidative stress and promote angiogenesis (37). The amount of matricellular cysteine-rich proteins Cyr61 and Cyr3, and connective tissue growth factor (CTGF) mRNAs were also greater in diabetic muscles. Cyr61 (CNN1) and CTGF (CNN2) are involved in ECM remodeling, and their proangiogenic activity suggests a role in the establishment and functioning of the vasculature (38). As a new finding based on the present results, it seems that in addition to traditional angiogenic growth factors, diabetes seems to alter the mRNA expression of several ECM proteins, which are involved in the regulation of blood vessel growth.

The effect of streptozotocin-induced type 1 diabetes and also type 2 diabetes on skeletal muscle gene expression has been studied earlier with microarrays (39, 40). Those studies focused mostly on energy metabolism and muscle structure, and none of the earlier studies have combined diabetes and exercise training. Decreased capillarization together with changes in angiogenic gene expression and responses to ischemia have recently been reported in leptin receptor-deficient mice, a model for type 2 diabetes (41). Albeit the differences in experimental set-ups, it is intriguing to find same genes, such as VEGF-A, VEGF-B, neuropilin-1, and Sparc, to be affected. Interestingly, similar changes in VEGF-A, Cyr61, CTGF, and metallothionein 2 mRNAs, as we found in diabetes, were observed in denervated mouse skeletal muscle (42). A common feature of these two models is a marked atrophy of muscle fibers.

Taken together, streptozotocin-induced diabetes and the subsequent hyperglycemia decreased the mRNA levels of proangiogenic proteins and increased those of antiangiogenic ones. This change of balance may be one of the major reasons for the markedly increased risk for peripheral cardiovascular complications in dia-

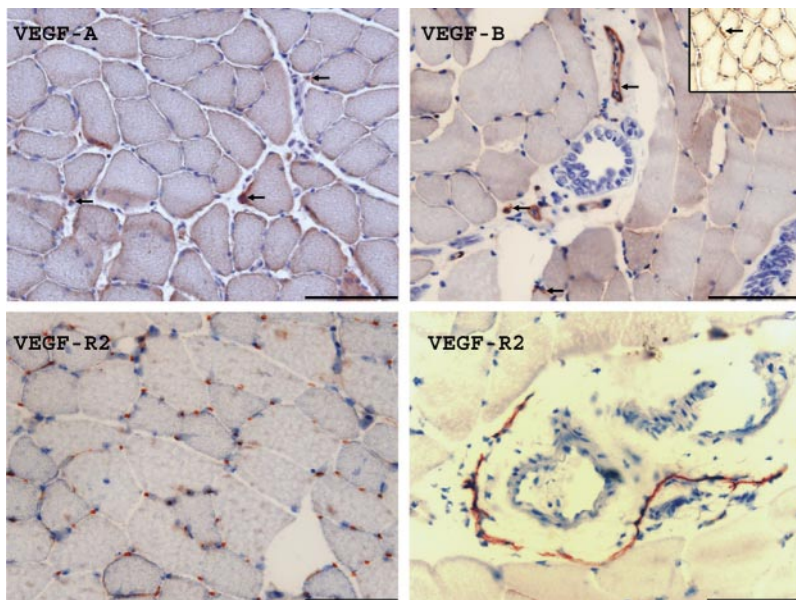


Figure 3. Localization of VEGF-A, VEGF-B and VEGFR-2 proteins in mouse skeletal muscle. VEGF-A protein was localized under sarcolemma in some of the muscle fibers, in the endothelium of larger blood vessels and in some capillaries (*upper left*), as has been reported also in earlier studies. As a new finding we show here, that VEGF-B was stained strongly in the larger blood vessels, in certain interstitial cells and in only few muscle fibers (*upper right*). Weak staining could be also observed in some capillaries. Inserted image shows the myofibrillar localization of VEGF-B. VEGFR-2 protein was expressed in most of the capillaries (*lower left*) and in vessels without thick smooth muscle cell layer (probably lymphatic vessels) situated close to arteries and large veins (*lower right*). There was no difference in the protein localization between the healthy and diabetic mice. Scale bars are 200 μm in other images except in the lower right image it is 100 μm .

betes. However, mRNAs of extracellular proteins, which have proangiogenic properties, were up-regulated in diabetic muscles and, thus, may induce compensatory protective events. In addition to the changes in mRNA levels, hyperglycemia induces the production of advanced glycation end-products (AGEs), which affect ECM degradation and angiogenesis (43). Because capillary network formation and maintenance is a complex process, the mechanisms by which diabetes affects skeletal muscle microvascular network potentially includes both the changes in the transcription of pro- and antiangiogenic factors and the remodeling of ECM (e.g., via production of AGEs).

The effectiveness of the exercise training in the present study was confirmed by the increased activity of citrate synthase in both healthy and diabetic trained mice (44). Also the blood Glc concentration tended to decrease in trained diabetic animals compared to sedentary. Acute exercise has been demonstrated to increase VEGF-A mRNA and protein levels markedly in healthy animal and human skeletal muscle (17, 18, 45). In healthy muscle this response is, however, attenuated when the training is continued (46, 47), whereas the structural changes, such as an increased number of capillaries, are observed at later stages (8). We did not observe significant differences in VEGF-A mRNA levels between healthy sedentary and healthy trained animals at any time point. This may be due to the time of sample collection (24 h after the last training bout), as the amount of VEGF-A mRNA has been shown to return to baseline values already after 8 h in rat skeletal muscle (17). Instead, the present results suggest that endurance exercise training induces favorable changes in the amount of mRNAs of several angiogenesis-related genes in diabetic muscles even up to 24 h after the last exercise bout. VEGF-A, VEGF-B, myoglobin, and SOD 2 mRNA levels were increased in trained diabetic mice compared with sedentary diabetic mice at least in some phase of the training period. In addition,

diabetes-induced increases in Hif-1 α and cysteine-rich proteins 3 and 61 were attenuated in the trained diabetic animals. Increases in VEGF-A, VEGF-B, and myoglobin mRNAs support enhanced capillary network and oxygen delivery to muscle fibers. Training also slightly reduced the diabetes-induced expression of metallothioneins, but also increased their expression after the first week of training in healthy animals. Metallothionein-1 and -2 have recently been demonstrated to be induced by acute exercise in human skeletal muscle and to stay elevated 24 h after the exercise (48). This was suggested to represent a mechanism whereby the muscle is protected against excess oxidative stress and injury.

Our results suggest that exercise training had substantially stronger beneficial effect on angiogenesis-related mRNA expression in diabetic than in control mice, as almost no changes were observed in the latter after 24 h (Table 2). However, exercise training could not completely restore the diabetes-induced changes back to the levels of healthy mice, although, it could significantly attenuate them. The increased amount of Hif-1 α mRNA in the sedentary diabetic mice is contradictory to the reduced VEGF-A expression, because Hif-1 α is known to up-regulate VEGF-A. This discrepancy may be due to the more important regulation of Hif-1 α at the protein concentration (49). In addition, in skeletal muscles also other stimuli, such as mechanical and shear stress to endothelial cells and myofibers, are possibly even more important in the regulation of VEGF-A expression (8, 50).

In our study the amount of VEGF-A protein was significantly reduced in sedentary diabetic muscles after 3 and 5 wk of diabetes, which is in line with the mRNA results. In trained diabetic mice the decrease was not significant until after five weeks. In line with the reduced levels of VEGF-A and other angiogenic factors, and the increased levels of angiogenesis inhibitors, capillary-to-fiber ratio was lower in diabetic mice com-

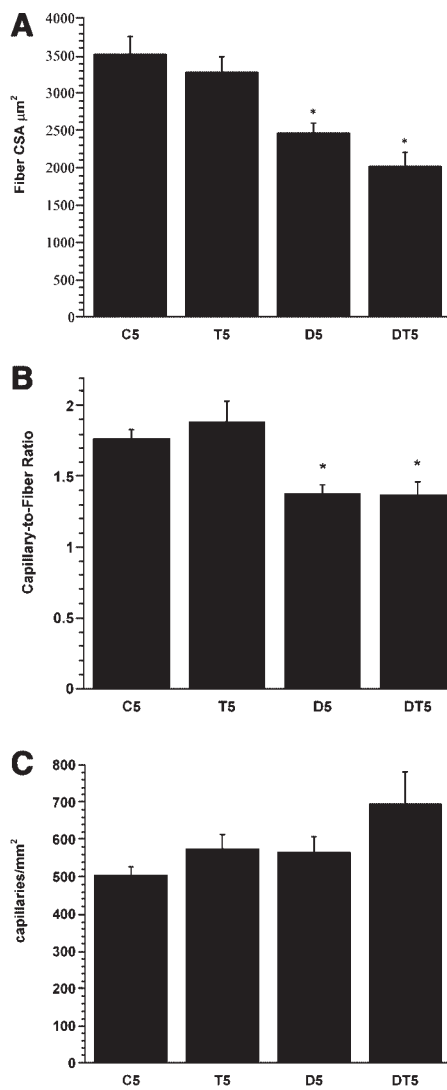


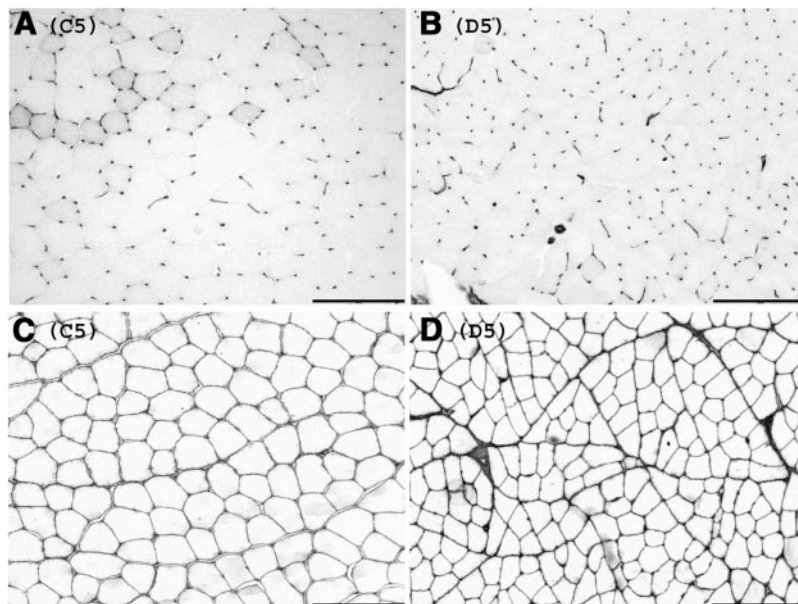
Figure 4. Muscle fiber cross-sectional area (A) and capillary-to-fiber ratio (B) were decreased in diabetic quadriceps femoris muscles after five weeks of diabetes and/or exercise training. Results are mean \pm SE. * $P < 0.05$ vs. healthy sedentary controls.

pared to healthy controls. Increased endothelial cell and myofiber apoptosis in diabetic muscles may also contribute to the reduced capillarization (51). Exercise training could not restore capillarization in diabetic mice, and the increase was not significant in healthy mice either. Capillarization was analyzed from the quadriceps femoris muscles, which may differ in response from commonly studied calf muscles gastrocnemius and soleus. Intramuscular gene therapy with VEGF-A or other angiogenic factors is thought to be one way of facilitating angiogenesis in ischemic skeletal muscle and the heart (51, 52). VEGF-A and tissue kallikrein gene therapy has been proven to be effective also in ischemic diabetic muscles in animal models (4, 51). Gene-transfer studies are often conducted in already ischemic muscles, but exercise training could probably work better as a preventive tool for peripheral vascular problems in diabetes.

Numerous studies have shown that type 1 diabetes is the predominant feature of mice and rats treated with streptozotocin (53), thus diabetes and subsequent hyperglycemia were presumably the main cause of the observed changes in the present study. Other nondiabetic effects cannot, however, be completely ruled out. It has been suggested that animal models can be used successfully to study specific aspects of the diabetic processes, but they should not be considered to represent the clinical disease (54, 55).

The present study showed that diabetes impairs capillarization and affects the expression of several genes involved in angiogenesis in skeletal muscle. Endurance training alleviated some of these changes but did not fully restore the diabetes-induced defects. These training effects, seen in the mRNA levels of angiogenesis-related genes, may be one of the mechanisms responsible for the beneficial effects of regular endurance exercise in diabetic patients. These data suggest that reduced skeletal muscle capillarization in

Figure 5. Representative images from healthy and diabetic skeletal muscles. Diabetes decreased muscle fiber area and capillary-to fiber ratio significantly. Capillaries are stained with CD31 antibody in images (A) (healthy, C5) and (B) (diabetic, D5). Muscle fibers are visualized with dystrophin staining in images (C) (C5) and (D) (D5). Scale bars are 200 μm .



type 1 diabetes is associated with the dysregulation of complex angiogenesis pathways. FJ

Ms. Aila Ollikainen is acknowledged for her excellent technical help. The Finnish Ministry of Education, LIKES Foundation for Promotion of Sport and Health Sciences, and Suomen Urheiluoopistosäätiö supported this study.

REFERENCES

1. Kannel, W. B., McGee, D. L. (1979) Diabetes and cardiovascular disease: the Framingham Study. *JAMA* **241**, 2035–2038
2. The Diabetes Control and Complications Trial Research Group (1993) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *NEJM* **329**, 977–986
3. Laakso, M., and Lehto, S. (1997) Epidemiology of macrovascular disease in diabetes. *Diabetes Rev.* **5**, 294–315
4. Rivard, A., Silver, M., Chen, D., Kearney, M., Magner, M., Annex, B., Peters, K., and Isner, J. M. (1999) Rescue of diabetes-related impairment of angiogenesis by intramuscular gene therapy with adeno-VEGF. *Am. J. Pathol.* **154**, 355–363
5. Abaci, A., Oguzhan, A., Kahraman, S., Eryol, N. K., Ünal, S., Arinc, H., and Ergin, A. (1999) Effect of diabetes mellitus on formation of coronary collateral vessels. *Circulation* **99**, 2239–2242
6. Sexton, W. L., Poole, D. C., and Mathieu-Costello, O. (1994) Microcirculatory structure-function relationship in skeletal muscle of diabetic rats. *Am. J. Physiol.* **266**, H1502–H1511
7. Kindig, C. A., Sexton, W. L., Fedde, M. R., and Poole, D. C. (1998) Skeletal muscle microcirculatory structure and hemodynamics in diabetes. *Respiration Phys.* **111**, 163–175
8. Hudlicka, O., Brown, M., and Egginton, S. (1992) Angiogenesis in skeletal and cardiac muscle. *Physiol. Rev.* **71**, 541–585
9. Leinonen, H., Matikainen, E., and Juntunen, J. (1982) Permeability and morphology of skeletal muscle capillaries in type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* **22**, 158–162
10. Wallenberg-Henriksson, H., Gunnarsson, R., Henriksson, J., Östman, J., and Wahren, J. (1984) Influence of physical training on formation of muscle capillaries in type I diabetes. *Diabetes* **33**, 851–857
11. Lash, J. M., Sherman, W. M., and Hamlin, R. L. (1989) Capillary basement membrane thickness and capillary density in sedentary and trained obese Zucker rats. *Diabetes* **38**, 854–860
12. Henriksson, J. (1992) Effects of physical training on the metabolism of skeletal muscle. *Diabetes Care* **15**, 1701–1711
13. Carmeliet, P. (2003) Angiogenesis in health and disease. *Nat. Med.* **9**, 653–660
14. Chou, E., Suzumi, I., Way, K. J., Opland, D., Clermont, A. C., Naruse, K., Suzuma, K., Bowling, N. L., Vlahos, C. J., Aiello, L. P., and King, G. L. (2002) Decreased cardiac expression of vascular endothelial growth factor and its receptors in insulin-resistant and diabetic states. *Circulation* **105**, 373–379
15. Sasso, F. C., Torella, D., Carbonara, O., Ellison, G. M., Torella, M., Scardone, M., Marra, C., Nasti, R., Marfella, R., Cozzolino, D., et al. (2005) Increased vascular endothelial growth factor expression but impaired vascular endothelial growth factor receptor signaling in the myocardium of type 2 diabetic patients with chronic coronary heart disease. *J. Am. Coll. Cardiol.* **46**, 827–834
16. Larger, E., Marre, M., Corvol, P., and Gasc, J.-M. (2004) Hyperglycemia-induced defects in angiogenesis in the chicken chorioallantoic membrane model. *Diabetes* **53**, 752–761
17. Breen, E. C., Johnson, E. C., Wagner, H., Tseng, H. M., Sung, L. A., and Wagner, P. D. (1996) Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. *J. Appl. Physiol.* **81**, 355–361
18. Gustafsson, T., Puntchart, A., Kaijser, L., Jansson, E., and Sundberg, C. J. (1999) Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. *Am. J. Physiol.* **276**, H679–H685
19. Redberg, R. F., Greenland, P., Fuster, V., Pyörälä, K., Blair, S. N., Folsom, A. R., Newman, A. B., O'Leary, D. H., Orchard, T. J., Psaty, B., et al. (2002) Prevention Conference VI: Diabetes and Cardiovascular Disease: Writing Group III: risk assessment in persons with diabetes. *Circulation* **105**, 144–152
20. Olefsky, J. M. (2001) Prospects for research in diabetes mellitus. *JAMA* **285**, 628–632
21. Gonzalez, C., Cuvellier, S., Hue-Beauvais, C., and Levi-Strauss, M. (2003) Genetic control of non-obese diabetic mice susceptibility to high-dose streptozotocin-induced diabetes. *Diabetologia* **46**, 1291–1295
22. Kainulainen, H., Ahomäki, E., and Vihko, V. (1984) Selected enzyme activities in mouse cardiac muscle during training and terminated training. *Basic Res. Cardiol.* **79**, 110–123
23. Jemiolo, B., and Trappe, S. (2004) Single muscle fiber gene expression in human skeletal muscle: validation of internal control with exercise. *Biochem. Biophys. Res. Co.* **320**, 1043–1050
24. Liu, W. M., Mei, R., Di, X., Ryder, T. B., Hubbell, E., Dee, S., Webster, T. A., Harrington, C. A., Ho, M. H., Baid, J., and Smeekens, S. P. (2002) Analysis of high density expression microarrays with signed-rank call algorithms. *Bioinformatics* **18**, 1593–1599
25. Hubbell, E., Liu, W. M., and Mei, R. (2002) Robust estimators for expression analysis. *Bioinformatics* **18**, 1585–1592
26. Lerman, O. Z., Galiano, R. D., Armour, M., Levine, J. P., and Gurtner, G. C. (2003) Cellular dysfunction in the diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to hypoxia. *Am. J. Pathol.* **162**, 303–312
27. Jiang, Z. Y., He, Z., King, B. L., Kuroki, T., Opland, D. M., Suzuma, K., Suzuma, I., Ueki, K., Kulkarni, R. N., Kahn, R., and King, G. L. (2003) Characterization of multiple signaling pathways of insulin in the vascular endothelial growth factor expression in vascular cells and angiogenesis. *J. Biol. Chem.* **278**, 31964–31971
28. Rissanen, T. T., Vajanto, I., Hiltunen, M. O., Rutanen, J., Kettunen, M. I., Niemi, M., Leppänen, P., Turunen, M. P., Markkanen, J. E., Arve, K., Alhava, E., Kauppinen, R. A., and Ylä-Herttuala, S. (2002) Expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 (KDR/Flk-1) in ischemic skeletal muscle and its regeneration. *Am. J. Pathol.* **160**, 1393–1403
29. Tammela, T., Enholm, B., Alitalo, K., and Paavonen, K. (2005) The biology of vascular endothelial growth factors. *Cardiovasc. Res.* **65**, 550–563
30. Van Weel, V., Deckers, M. L., Grimbergen, J. M., van Leuven, K. J. M., Lardenoye, J. H. P., Schlingemann, R. O., van Nieuw Amerongen, G. P., van Bockel, J. H., van Hinsbergh, V. W. M., and Quax, P. H. A. (2004) Vascular endothelial growth factor overexpression in ischemic skeletal muscle enhances myoglobin expression in vivo. *Circ. Res.* **95**, 58–66
31. Williams, R. S., and Annex, B. H. (2004) Plasticity of myocytes and capillaries: a possible coordinating role for VEGF. *Circ. Res.* **94**, 7–8
32. Brownlee, M. (2001) Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**, 813–820
33. Jiménez, B., Volpiert, O. V., Crawford, S. E., Febbraio, M., Silverstein, R. L., and Bouck, N. (2000) Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat. Med.* **6**, 41–48
34. Claudio, P. P., Stiegler, P., Howard, C. M., Bellan, C., Minimo, C., Tosi, G. M., Rak, J., Kovatich, A., De Facio, P., Micheli, P., et al. (2001) RB2/p130 Gene-enhanced expression down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in vivo. *Cancer Res.* **61**, 462–468
35. Stenina, O., Krukovets, I., Wang, K., Zhou, Z., Forudi, F., Penn, M. S., Topol, E. J., and Plow, E. F. (2003) Increased expression of thrombospondin-1 in vessel wall of diabetic Zucker rat. *Circulation* **107**, 3209–3215
36. Sage, E. H., Reed, M., Funk, S. E., Truong, T., Steadale, M., Puolakkainen, P., Maurice, D. H., and Bassuk, J. A. (2003) Cleavage of the extracellular matrix protein SPARC by matrix metalloproteinase 3 produces polypeptides that influence angiogenesis. *J. Biol. Chem.* **278**, 37849–37857
37. Giralt, M., Penkowa, M., Lago, N., Molinero, A., and Hidalgo, J. (2002) Metallothionein-1+2 protect the CNS after focal brain injury. *Exp. Neurol.* **173**, 114–128
38. Perbal, B. (2004) CNN proteins: multifunctional signalling regulators. *Lancet* **363**, 62–64

39. Yechoor, V. K., Patti, M. E., Saccone, R., and Kahn, C. R. (2002) Coordinated patterns of gene expression for substrate and energy metabolism in skeletal muscle of diabetic mice. *Proc. Nat. Acad. Sci.* **99**, 10587–10592
40. Sreekumar, R., Halvatsiotis, P., Schimke, J. C., and Nair, K. S. (2002) Gene expression profile in skeletal muscle of type 2 diabetes and the effects of insulin treatment. *Diabetes* **51**, 1913–1920
41. Schiekofer, S., Galasso, G., Sato, K., Kraus, B. J., and Walsh, K. (2005) Impaired revascularization in a mouse model of type 2 diabetes is associated with dysregulation of a complex angiogenic-regulatory network. *Arterioscler. Thromb. Vasc. Biol.* **25**, 1603–1609
42. Magnusson, C., Svensson, A., Christerson, U., and Tägerud, S. (2005) Denervation-induced alterations in gene expression in mouse skeletal muscle. *Eur. J. Neurosci.* **21**, 577–580
43. Tamarat, R., Silvestre, J. S., Huijberts, M., Benessiano, J., Ebrahimian, T. G., Duriez, M., Wautier, M.-P., Wautier, J. L., and Levy, B. I. (2003) Blockade of advanced glycation end-product formation restores ischemia-induced angiogenesis in diabetic mice. *Proc. Nat. Acad. Sci.* **100**, 8555–8560
44. Holloszy, J. O., and Booth, F. W. (1976) Biochemical adaptation to endurance exercise in muscle. *Annu. Rev. Physiol.* **38**, 273–291
45. Gustafsson, T., Knutson, A., Puntchart, A., Kaijser, L., Sandberg-Nordqvist, A. C., Sundberg, C. J., and Jansson, E. (2002) Increased expression of vascular endothelial growth factor in human skeletal muscle in response to short-term one-legged exercise training. *Pflügers Arch.* **444**, 752–759
46. Gavin, T. P., and Wagner, P. D. (2001) Effect of short-term exercise training on angiogenic growth factor gene responses in rats. *J. Appl. Physiol.* **90**, 1219–26
47. Richardson, R. S., Wagner, H., Mudaliar, S. R. D., Saucedo, E., Henry, R., and Wagner, P. D. (2000) Exercise adaptation attenuates VEGF gene expression in human skeletal muscle. *Am. J. Physiol.* **279**, H772–H778
48. Penkowa, M., Keller, P., Keller, C., Hidalgo, J., Giralt, M., and Pedersen, B. K. (2005) Exercise-induced methallothionein expression in human skeletal muscle fibers. *Exp. Physiol.* **90**, 477–486
49. Ameln, H., Gustafsson, T., Sundberg, C. J., Okamoto, K., Jansson, E., Poellinger, L., and Makino, Y. (2005) Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. *FASEB J.* **19**, 1009–1011
50. Hudlicka, O. (1991) What makes blood vessels grow? *J. Physiol.* **444**, 1–24
51. Emanuelli, C., Graiani, G., Salis, M. B., Gadau, S., Desortes, E., and Madeddu, P. (2004) Prophylactic gene therapy with human tissue kallikrein ameliorates limb ischemia recovery in type 1 diabetic mice. *Diabetes* **53**, 1096–1103
52. Rissanen, T. T., Markkanen, J. E., Gruchala, M., Heikura, T., Puranen, A., Kettunen, M. I., Kholova, I., Kauppinen, R. A., Achen, M. G., Stacker, S. A., *et al.* (2003) VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. *Circ. Res.* **92**, 1098–1106
53. Rees, D. A., and Alcolado, J. C. (2005) Animal models of diabetes mellitus. *Diabet. Med.* **22**, 359–370
54. Roep, B. O., and Atkinson, M. (2004) Animal models have little to teach us about type 1 diabetes: 1. In support of this proposal. *Diabetologia* **47**, 1650–1656
55. Leiter, E. H., and von Herrath, M. (2004) Animal models have little to teach us about type 1 diabetes: 1. In opposition of this proposal. *Diabetologia* **47**, 1657–1660

*Received for publication August 24, 2005.
Accepted for publication January 19, 2006.*

Effects of experimental type 1 diabetes and exercise training on angiogenic gene expression and capillarization in skeletal muscle

Riikka Kivela,^{*,†,1} Mika Silvennoinen,^{*,†} Anna-Maria Touvra,[†]
T. Maarit Lehti,^{*} Heikki Kainulainen,^{*,†} and Veikko Vihko^{*}

^{*}LIKES Research Center for Sport and Health Sciences, Jyväskylä, Finland; and [†]Neuromuscular Research Center, Department of Biology of Physical Activity, University of Jyväskylä, Jyväskylä, Finland



To read the full text of this article, go to <http://www.fasebj.org/cgi/doi/10.1096/fj.05-4780fje>

SPECIFIC AIMS

Angiogenesis is a process of new blood microvessel formation and it is regulated by pro- and antiangiogenic factors. Many physiological and pathological stimuli affect the expression of these factors. In diabetic skeletal muscles, angiogenesis has been shown to be impaired; whereas in healthy muscles, exercise increases the production of angiogenic growth factors and promotes the growth of new capillaries. The purpose of the present study was to investigate the changes in angiogenic gene expression and capillarization in diabetic skeletal muscle and to study whether exercise could reverse these changes.

PRINCIPAL FINDINGS

1. Streptozotocin-induced type 1 diabetes reduced the mRNA expression of angiogenic growth factors in mouse skeletal muscle

Diabetic and healthy animals were randomly assigned into 12 groups ($n = 5$ per group), which were either sedentary or trained for 1, 3, or 5 weeks. Training groups performed 1 h per day of treadmill running at 21 m/min and at an uphill incline of 2.5° for 5 days a week. Mice were killed 24 h after the last training session to focus on the effects of training and not the effects resulting from the last exercise bout. Oligonucleotide array analyses (Affymetrix Gene Chip MG U74Av2) and real-time PCR were performed to compare mRNA expression of angiogenesis-related genes in healthy and diabetic mice and the effects of exercise training. Diabetes decreased the mRNA levels of the major angiogenic growth factor, vascular endothelial growth factor (VEGF)-A, by 30–50% depending on time point (Fig. 1). The levels of VEGF-B, neuropilin-1, VEGF receptor-1 (Flt-1) and VEGF receptor-2 (Flk-1)

were significantly reduced as well. Diabetes also down-regulated the expression of myoglobin and MnSOD.

2. Reduced VEGF-A protein content in diabetic muscles, but no change in VEGF-A, VEGF-B or VEGFR-2 protein localization

VEGF-A protein concentration in skeletal muscle homogenates was reduced in sedentary diabetic mice after 3 and 5 weeks of diabetes compared with the healthy sedentary mice ($P < 0.05$). In the endurance-trained diabetic groups, VEGF-A protein content was significantly decreased only after 5 weeks. After 3 weeks, trained diabetic animals tended to have higher VEGF-A content than sedentary diabetic mice ($P = 0.086$). We found no significant change in VEGF-A content in healthy trained mice compared with the healthy sedentary group.

3. The mRNA expression of angiogenesis inhibitors and of metallothioneins was increased by diabetes

The amount of thrombospondin-1 (TSP-1) mRNA, a known inhibitor of angiogenesis, was markedly increased in diabetic muscles (Fig. 1) as well as the concentration of retinoblastoma-like-2 (Rbl-2), which is considered a potent VEGF inhibitor. Diabetes also increased markedly the expression of metallothionein-1 and -2 at all time points. In diabetic muscles, Hif-1 α mRNA levels were also higher than in healthy mice.

4. Increased mRNA expression of CNN family proteins

Extracellular matrix (ECM) remodeling is an important step in angiogenesis. The mRNA levels of matricel-

¹Correspondence: LIKES Research Center, Rautpohjankatu 8a, Jyväskylä FIN-40740, Finland. E-mail: riikka.kivela@likes.fi
doi: 10.1096/fj.05-4780fje

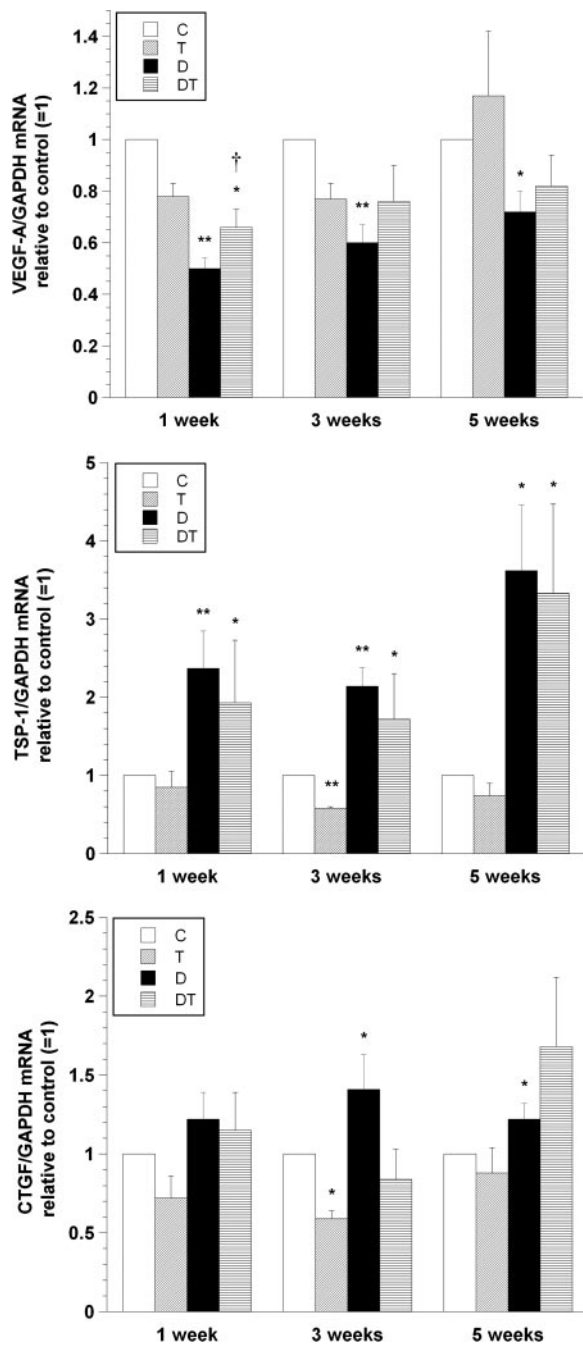


Figure 1. Fold-change of VEGF-A, thrombospondin-1 (TSP-1), and connective tissue growth factor (CTGF) mRNA content in trained healthy (diagonally striped bars), sedentary diabetic (black bars) and trained diabetic (horizontally striped bars) mouse calf muscles compared to the sedentary healthy controls (white bars, set to value 1). Values are mean \pm SE. * $P < 0.05$ vs. healthy sedentary controls, $\dagger P < 0.05$ vs. respective sedentary diabetic (=effect of training). Note the varying scale on the Y-axis due to the magnitude and direction of the changes.

lular proteins, connective tissue growth factor (CTGF), and cysteine-rich protein 61 (Cyr61) were increased in diabetic muscles (Fig. 1). These proteins belong to the CNN protein family and are involved in ECM remodeling and angiogenesis.

5. Effects of exercise training on diabetes-induced changes in gene expression

Endurance training alleviated the diabetes-induced changes in the mRNA levels of VEGF-A, VEGF-B, neuropilin-1, myoglobin, MnSOD, metallothioneins, and Hif-1 α . However, exercise did not significantly decrease the levels of angiogenesis inhibitors TSP-1 or Rbl-2.

6. Decreased capillary-to-fiber ratio and muscle fiber cross-sectional area in diabetic mice

Both sedentary and trained diabetic mice showed a significant decrease in muscle fiber cross-sectional area (CSA) after 5 weeks compared with healthy controls ($P < 0.05$). Capillary density (cap - mm⁻²) was not significantly changed due to diabetes, although it tended to be increased due to the reduced fiber cross-sectional area. Instead, the capillary-to-fiber ratio, which is commonly used to describe capillary supply to muscle fibers, was significantly decreased in both diabetic groups compared to the healthy control group ($P < 0.05$) but was not significantly different between the trained and sedentary groups in either diabetic or healthy mice.

DISCUSSION AND SIGNIFICANCE

In the present study we determined separate and combined effects of diabetes and exercise training on the expression of pro- and antiangiogenesis genes and proteins and on the capillarization in mouse skeletal muscle. The high blood glucose (Glc) concentration and considerable loss of body wt confirmed that streptozotocin had induced diabetes in the studied mice.

Diabetes decreased the mRNA concentration of many genes known to be involved in the regulation of angiogenesis, most interestingly those of VEGF-A and VEGF-B together with their receptors VEGFR-1/Flt1, VEGFR-2/Flk1, and neuropilin-1. The effect of diabetes on the VEGF-B and VEGF receptor mRNAs in skeletal muscle has not been reported earlier. In diabetic rabbits a reduction in VEGF-A mRNA levels in ischemic skeletal muscle has been observed previously. In cell cultures high Glc levels have been reported to inhibit VEGF-A production and insulin, in turn, to enhance it. In the present study, the amount of VEGF-A protein was also significantly reduced in sedentary diabetic muscles after 3 and 5 weeks of diabetes, which is in line with the mRNA results.

In addition to the mRNAs, we studied the localization of three important angiogenesis proteins—VEGF-A, VEGFR-2 and VEGF-B—in healthy and diabetic muscles. VEGF-A localized to myofibers and endothelial cells and VEGFR-2 to endothelial cells as has been shown earlier. To our knowledge, the localization of VEGF-B protein in skeletal muscle has not been reported earlier. The present findings showed that VEGF-B is localized mainly in larger blood vessels, capillaries, and interstitial cells. Similarly to VEGF-A, it

was also detectable under the sarcolemma in a few muscle fibers. The present results confirm the presence of both VEGF-B mRNA and protein in skeletal muscle, and its localization to blood vessels supports its role in the maintenance of blood vessel endothelium. The localization of these three proteins was similar in healthy and diabetic skeletal muscle.

Diabetes also increased mRNA levels of several genes involved in angiogenesis. The most significant of these were the increases in TSP-1 and Rbl-2, both of which are known to inhibit angiogenesis. Increased expression of TSP-1 in the vessel wall of diabetic Zucker rats has been reported earlier, and it was proposed that it could be a direct response of vascular cells to Glc and, thus, a link between diabetes and atherosclerotic complications. The present results extend this finding from aorta to peripheral muscle tissues. Overexpression of retinoblastoma-like 2 (Rb2/Rbl2) decreased VEGF-A mRNA and protein levels in two tumor cell types, together with inhibited tumor angiogenesis, which suggest that Rbl2 is a potent VEGF-A inhibitor. In our experiment, diabetic muscles expressed higher mRNA levels of retinoblastoma-like 2 and lower mRNA levels of VEGF-A than healthy muscles.

Stress-inducible metallothionein-1 and -2 mRNA levels were more than 10-fold higher after the first week of diabetes. Thereafter, the levels slightly attenuated but remained still elevated throughout the whole experiment. The amounts of extracellular matrix proteins Cyr61, Cyr3, and connective tissue growth factor (CTGF) mRNAs were also greater in diabetic muscles. Cyr61 (CNN1) and CTGF (CNN2) are involved in ECM remodeling, and their proangiogenic activity suggests a role in the establishment and functioning of the vasculature. As a new finding based on the present results, it seems that, in addition to traditional angiogenic growth factors, diabetes could alter the mRNA expression of several ECM proteins, which are involved in the regulation of blood vessel growth.

VEGF-A, VEGF-B, myoglobin, and superoxide dismutase 2 mRNA levels were increased in trained diabetic mice compared with sedentary diabetic mice at least in some phase of the training period. In addition, diabetes-induced increases in Hif-1 α and cysteine-rich proteins 3 and 61 were attenuated in the trained diabetic animals. Increases in VEGF-A, VEGF-B, and myoglobin mRNAs support enhanced capillary network and oxygen delivery to muscle fibers. Training also slightly reduced the diabetes-induced expression of metallothioneins but also increased their expression after the first week of training in healthy animals.

In line with the reduced levels of VEGF-A and other proangiogenic factors, and the increased levels of an-

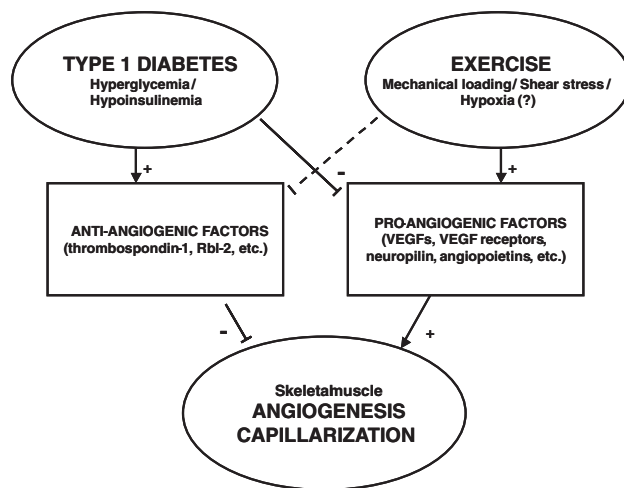


Figure 2. A schematic summary based on the existing literature and the findings of the present study. Type 1 diabetes impairs skeletal muscle capillarization and/or angiogenesis by reducing the mRNA concentration of pro-angiogenic factors and increasing the expression of antiangiogenic factors. Exercise training induces opposite effect on pro-angiogenic factors, which in the present experimental model did not completely compensate the diabetes-induced changes. Effects of exercise training on antiangiogenic factors have not been studied earlier and in the present experiment it could not reverse the increased expression in diabetic muscles (dashed line). In the figure, increased expression or activation is marked with + and decreased expression or inhibition with -.

giogenesis inhibitors, capillary-to-fiber ratio was lower in diabetic mice compared with healthy controls. Exercise training could not restore capillarization in diabetic mice, and the training-induced increase was not statistically significant in healthy mice either.

In conclusion, the present study showed that streptozotocin-induced diabetes and the subsequent hyperglycemia reduce the mRNA levels of proangiogenic proteins and increase those of antiangiogenic ones, together with decreased capillarization. This change of balance may be one of the major reasons for the markedly increased risk for peripheral cardiovascular complications in diabetes. Endurance training alleviated some of these changes but did not fully restore the diabetes-induced defects. These training effects, seen in the mRNA levels of angiogenesis-related genes, may be one of the mechanisms responsible for the beneficial effects of regular endurance exercise in diabetic patients. These data suggest that reduced skeletal muscle capillarization in type 1 diabetes is associated with the dysregulation of complex angiogenesis pathways (Fig. 2).