Effects of acute exercise, exercise training, and diabetes on the expression of lymphangiogenic growth factors and lymphatic vessels in skeletal muscle

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Kivela R, Silvennoinen M, Lehti M, Kainulainen H, Vihko V. Effects of acute exercise, exercise training, and diabetes on the expression of lymphangiogenic growth factors and lymphatic vessels in skeletal muscle. Am J Physiol Heart Circ Physiol 293: H2573–H2579, 2007. First published August 31, 2007; doi:10.1152/ajpheart.00126.2007.—Blood and lymphatic vessels together form the circulatory system, allowing the passage of fluids and molecules within the body. Recently we showed that lymphatic capillaries are also found in the capillary bed of skeletal muscle. Exercise is known to induce angiogenesis in skeletal muscle, but it is not known whether exercise has effects on lymphangiogenesis or lymphangiogenic growth factors. We studied lymphatic vessel density and expression of the main lymphangiogenic growth factors VEGF-C and VEGF-D and their receptor VEGFR-3 in response to acute running exercise and endurance exercise training in the skeletal muscle of healthy and diabetic mice. VEGF-C mRNA expression increased after the acute exercise bout (P < 0.05) in healthy muscles, but there was no change in diabetic muscles. VEGF-C levels were not changed either in healthy or in diabetic muscle after the exercise training. Neither acute exercise nor exercise training had an effect on the mRNA expression of VEGF-D or VEGFR-3 in healthy or diabetic muscles. Lymphatic vessel density was similar in sedentary and trained mice and was >10-fold smaller than blood capillary density. Diabetes increased the mRNA expression of VEGF-D (P < 0.01). Increased immunohistochemical staining of VEGF-D was found in degenerative muscle fibers in the diabetic mice. In conclusion, the results suggest that acute exercise or exercise training does not significantly affect lymphangiogenesis in skeletal muscle. Diabetes increased the expression of VEGF-D in skeletal muscle, and this increase may be related to muscle fiber damage.

Lymphangiogenesis; vascular endothelial growth factor-C; vascular endothelial growth factor-D; vascular endothelial growth factor receptor-3; messenger RNA

Lymphatic vessels are part of the circulatory system, and they function in close relation with blood vessels. Fluids, macromolecules, and migrating inflammatory cells pass through the blood capillary endothelia, enter the tissues, and are subsequently absorbed into the lymphatic vessels. The formed lymph fluid is transported via initial lymphatic capillaries to collecting vessels, to lymph nodes, and finally back to the blood.

Blood flow in skeletal muscles increases markedly during exercise (27), and this exercise-induced hyperemia results in increased net capillary filtration. Increased filtration, in turn, increases hydrostatic and colloid osmotic pressure in the interstitial fluid and addresses the need for increased lymph flow to maintain optimal conditions in the muscle (2). Indeed, exercise has been shown to increase skeletal muscle lymph flow markedly in both animal (3, 8, 17) and human (14, 15, 24) studies, especially at the onset of exercise. We recently showed (20) that skeletal muscles contain small capillary-sized lymphatic vessels, which are located next to blood capillaries between muscle fibers but are much fewer in number.

The increased number of capillaries after long-term endurance exercise training and electrical stimulation is an established and well-described phenomenon in human and animal skeletal muscle (reviewed in Ref. 16). Angiogenic signals are mediated by a number of growth factors and cytokines, and the balance between positive and negative regulators determines the growth or regression of blood vessels (7). Similar mechanisms are thought to be involved in lymphangiogenesis. Much less, however, is known about this part of the circulation.

The vascular endothelial growth factor (VEGF) family of proteins is one of the most important players in angiogenesis and lymphangiogenesis. It consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PIGF), which act as ligands for receptors VEGFR-1, VEGFR-2, and VEGFR-3 (9, 30). VEGF-A is able to bind to and activate VEGFR-1 and -2 receptors, and it is considered the main growth factor for physiological and pathological angiogenesis. VEGF-B binds only to VEGFR-1, and it is suggested to modulate the biological activity of VEGF-A. VEGF-C and VEGF-D are primary lymphangiogenic factors, inducing the growth of lymphatic vessels during development and in adults (30). Full-length VEGF-C and VEGF-D preferentially bind to VEGFR-3, inducing lymphangiogenesis. Further proteolytic processing of these proteins enables them to induce angiogenesis by activating VEGFR-2 in humans (31). In mice, VEGF-C activates both VEGFR-2 and -3, as in humans, but VEGF-D activates only VEGFR-3 (4).

An acute exercise bout has been shown to induce upregulation of angiogenic genes, especially VEGF-A, in skeletal muscle (6, 10, 12, 13). Increased mRNA and protein levels of VEGF-A have been linked to greater capillarization in muscles after exercise training. The positive effects of exercise on blood vessel growth in skeletal muscle are similar to, e.g., the effects of gene therapy, which can also enhance lymphangiogenesis. However, no studies on the effects of exercise on lymphangiogenic growth factors or lymphangiogenesis have been reported. Diabetes affects the peripheral vascular bed, and in skeletal muscle diabetes has opposite effects from exercise training and electrical stimulation is an established and well-described phenomenon in human and animal skeletal muscle (reviewed in Ref. 16). Angiogenic signals are mediated by a number of growth factors and cytokines, and the balance between positive and negative regulators determines the growth or regression of blood vessels (7). Similar mechanisms are thought to be involved in lymphangiogenesis. Much less, however, is known about this part of the circulation.

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lymphatic vessel density and expression of the lymphangiogenic growth factors VEGF-C and VEGF-D and their receptor VEGFR-3 in the skeletal muscle of healthy and diabetic mice.

**MATERIALS AND METHODS**

**Animals and Experimental Setup**

All experimental procedures were approved by the Animal Care and Use Committee of the University of Jyväskylä. Mice were housed under standard conditions (temperature 22°C, humidity 60 ± 10%, light from 8:00 AM to 8:00 PM), and they had free access to tap water and food pellets (R36, Labfor, Stockholm, Sweden). This study consists of two experiments: experiment 1 was an acute exercise bout, and experiment 2 was an exercise training study.

**Experiment 1.** Ten- to fifteen-week-old male NMRI mice (n = 48, Harlan) were used for the study. Mice were randomly assigned into healthy and diabetic groups. The diabetic group received a single peritoneal injection of streptozotocin (180 mg/kg; Sigma-Aldrich) dissolved in a sodium citrate buffer solution (0.1 mol/l, pH 4.5) to induce Type 1 diabetes. The other group received a sham injection of an equal volume of the buffer. Diabetes was confirmed 3 days after the injection by a blood glucose test (HemoCue B-Glucose analyzer, Angelholm, Sweden). The mice were not treated with insulin. Ten days after the injections the mice were divided into three healthy and three diabetic groups (n = 8). One healthy and one diabetic group served as sedentary control groups (H and D), and four groups performed a 1-h running exercise on a treadmill (speed 21 m/min with an uphill incline of 2.5°). One healthy and one diabetic exercise group were killed 3 h after (HE3 and DE3) and the other two groups 6 h after (HE6 and DE6) the exercise. Before the experiment, all mice were familiarized with treadmill running.

**Experiment 2.** Diabetes was induced in 10- to 15-wk-old male NMRI mice as described above (n = 60). Diabetes was confirmed 72 h after the injection by a urine glucose test (Glukotest, Roche), and the mice were not treated with insulin during the experiment. Diabetic and healthy animals were randomly assigned into 12 groups, which were either sedentary or trained for 1, 3, or 5 wk. The groups were 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). The training groups performed 1 h/day of treadmill running at 21 m/min and an uphill incline of 2.5° for 5 days/wk. The same person always conducted the training sessions. Trained mice together with their sedentary controls were killed 24 h after the last training bout.

**Tissue Preparation, Blood Glucose Concentration, and Citrate Synthase Activity**

Calf muscles (soleus, gastrocnemius, and plantaris) were removed, snap frozen in liquid nitrogen, and stored at −80°C for further analysis. In experiment 2, a middle part of quadriceps femoris muscle (MQF) was mounted in an OCT embedding medium (Miles, Elkhart, IN) under a microscope to orientate muscle fibers vertically and snap frozen in isopentane (−160°C) cooled with liquid nitrogen.

In experiment 1 blood glucose was measured from the whole blood, and in experiment 2 serum was separated. Glucose concentration was analyzed with a HemoCue B-Glucose analyzer at the end of the experiments. Citrate synthase activity to estimate the effects of exercise training was analyzed from the calf muscles in experiment 2 as described previously (21).

**RNA Extraction**

Total RNA was isolated from gastrocnemius muscle in experiment 1 and from the calf muscle complex containing gastrocnemius, soleus, and plantaris muscles in experiment 2 with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The concentration and purity of RNA were determined by spectrophotometry at wavelengths of 260 and 280 nm. Integrity was checked with agarose gel electrophoresis. RNA was reverse-transcribed to cDNA with a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA).

**Real-Time Quantitative PCR**

The ABI Prism Sequence Detection System 7700 (Applied Biosystems) was used to perform SYBR Green (experiment 1)- or TaqMan probe (experiment 2)-based real-time PCR reactions. TaqMan primer and probe sets were designed and synthesized by Applied Biosystems. Primers for SYBR Green reactions were adopted from Thijssen et al. (32) and produced by TAG Copenhagen (Copenhagen, Denmark). Assay numbers and primer sequences are presented in Table 1. Target genes in the sample were quantified according to the corresponding gene-specific standard curve. All samples were analyzed in triplicate. As an endogenous control to correct for potential variation in RNA loading, GAPDH mRNA was used. GAPDH is considered the most stable internal control in exercise studies (18), and in the microarray analyses of the samples from experiment 2 GAPDH showed the steadiest expression in all conditions when the normally used housekeeping genes were compared (21).

**Lymphatic Vessel Density**

Lymphatic vessel density was analyzed from healthy and diabetic sedentary (C5 and D5) and exercise-trained (T5 and DT5) mice. Transverse sections (10 μm) of MQF were cut in a cryostat at −25°C. The sections were air dried, fixed in cold acetone, and incubated with 3% BSA in PBS. Goat anti-human LYVE-1 (R&D Systems, Minneapolis, MN) primary antibody with Alexa Fluor 555-labeled secondary antibody (Molecular Probes, Eugene, OR) were used to detect lymphatic endothelium. Blood capillaries were stained with isocititin GS-Ba labeled with Alexa Fluor 488 (Molecular Probes). Lymphatic capillaries per square millimeter were counted with AnalySIS software (Olympus, Tokyo, Japan). Only transversely cut capillaries (both blood and lymphatic) were counted, and the large lymphatic

Table 1. Primers and probes for real-time quantitative PCR

<table>
<thead>
<tr>
<th>GenBank Accession No.</th>
<th>SYBR Green Primers (5’–3’)</th>
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<tbody>
<tr>
<td>VEGF-C Mm00437313_m1</td>
<td>NM_010228</td>
</tr>
<tr>
<td>VEGF-D Mm00438965_m1</td>
<td>NM_010612</td>
</tr>
<tr>
<td>VEGFR-3 Mm00433337_m1</td>
<td>XM_129720</td>
</tr>
<tr>
<td>GAPDH Mm99999915_m1</td>
<td>NM_008084.2</td>
</tr>
</tbody>
</table>

TaqMan probe-based assays are gene expression assays from Applied Biosystems. Primer sequences for SYBR Green are adopted from Thijssen et al. (32). VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.
vessels next to arteries and veins were excluded. Several images from different parts of the muscle were analyzed to cover most of the sample.

**Immunohistochemistry**

Transverse sections of MQF (n = 10 healthy and 10 diabetic) were air dried, fixed in cold acetone, and incubated with 5% blocking serum. Primary antibodies (goat anti-mouse VEGF-C (Santa Cruz Biotechnology, Santa Cruz, CA), VEGF-D (R&D Systems), and VEGFR-3 (kindly provided by Prof. Kari Alitalo, University of Helsinki, Helsinki, Finland)) were visualized with an avidin-biotin peroxidase kit (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) using 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO) as a chromogen. The sections were counterstained with Mayer’s hematoxylin. Negative controls were done by omitting the primary antibody or using an irrelevant antibody of the same isotype.

**VEGF-D ELISA**

VEGF-D protein concentration was analyzed from serum and skeletal muscle samples from experiment 2 (n = 59). 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). VEGF-D was determined from serum samples and muscle homogenates by a commercial ELISA kit according to the manufacturer’s instructions (Mouse VEGF-D DuoSet, R&D Systems). VEGF-D levels were obtained by a microplate reader at 450 nm and corrected by readings at 540 nm. VEGF-D concentration in muscle homogenates was normalized against the total protein concentration of the homogenate. Dissolved muscle protein concentration was measured with the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA).

**Statistical Analysis**

Statistical analyses were carried out with SPSS for Windows statistical software (release 13.0; SPSS, Chicago, IL). All data were analyzed for normality. Depending on the distribution of the data, analysis of variance (ANOVA) or nonparametric Kruskal-Wallis test with Mann-Whitney U-tests was used to analyze differences between groups. The significance level was set at P < 0.05. Data are presented as means ± SD. GAPDH-normalized mRNA expressions are reported as a fold change from a healthy control group, which is set to 1.

**RESULTS**

**Glucose Concentration and Body Mass**

**Experiment 1.** Whole blood glucose was significantly higher in diabetic mice (35.8 ± 6.1 vs. 8.1 ± 1.1 mmol/l; P < 0.01). Diabetic and healthy mice did not differ in body weight at the beginning of the experiment (36.8 ± 2.3 vs. 36.9 ± 3.2 g), but after 10 days of diabetes the diabetic mice weighed significantly less than the healthy mice (33.1 ± 4.1 vs. 37.9 ± 3.3 g; P < 0.001).

**Experiment 2.** Diabetic mice had significantly higher serum glucose levels (52.7 ± 6.6 vs. 10.0 ± 1.4 mmol/l; P < 0.001), and their body mass was reduced at the end of the experiment (pre 41.7 ± 0.8 vs. post 31.4 ± 3.5 g; P < 0.001). No change was observed in the body weight of healthy mice during the study period. Citrate synthase activity was higher in both healthy and diabetic exercise-trained groups than in their respective sedentary groups (P < 0.001–0.05).

**Messenger RNA**

**Experiment 1.** Skeletal muscle VEGF-C mRNA expression was lower in the healthy control group than in the healthy exercised group and all the diabetic groups (P < 0.01–0.05) (Fig. 1). VEGF-C expression in exercised diabetic mice did not differ from that in their respective control group. Exercise had no effect on VEGF-D mRNA expression in healthy or diabetic mice. However, VEGF-D expression was significantly greater in DE3 and DE6 groups than in their respective healthy groups, HE3 and HE6 (P < 0.01), and in the healthy control group (P < 0.05). VEGFR-3 mRNA expression was affected neither by exercise nor by diabetes.

**Experiment 2.** After 1, 3, or 5 wk, there were no effects of exercise training on the mRNA expression levels of VEGF-C, VEGF-D, or VEGFR-3 in healthy or diabetic mice (Fig. 2). VEGF-D expression was significantly increased in the diabetic mice compared with the healthy mice at all studied time points (P < 0.01). The variation in the diabetic groups was large, because individual responses varied from 1.2- to 18.9-fold increase compared with the respective controls. After 1 and 3 wk of training, VEGF-D expression was lower in the trained diabetic groups than in the respective sedentary diabetic groups (P < 0.05). Diabetes did not affect the expression of VEGF-C or VEGFR-3 mRNA. In both experiments the mRNA levels of VEGF-D correlated positively with blood glucose levels (experiment 1: r = 0.567, experiment 2: r = 0.549; P < 0.001).

**Fig. 1.** Vascular endothelial growth factor (VEGF)-C (A), VEGF-D (B), and VEGF receptor (VEGFR)-3 (C) mRNA levels (normalized to GAPDH) in healthy and diabetic skeletal muscle 3 and 6 h after a 1-h running exercise (experiment 1). Expression of VEGF-C mRNA was greater in exercised healthy mice and in all diabetic mice than in healthy control mice (A). In diabetic mice, the expression of VEGF-D was increased significantly compared with healthy mice (B). No change was observed in VEGFR-3 (C). The values are means ± SD. **P < 0.01, *P < 0.05 vs. healthy control; ###P < 0.001, ##P < 0.01 vs. respective healthy exercised group (n = 8 in each group).
Lymphatic Vessel Density

Lymphatic capillaries were not evenly distributed throughout the MQF muscle group (Fig. 3). Some areas had markedly more small lymphatic capillaries than others. The mean lymphatic capillary density in the studied mice varied from 17 to 54 capillaries/mm², whereas the blood capillary density was from 510 to 718 capillaries/mm². These numbers do not include larger lymphatic vessels in the perimysium or arterioles and veins. No differences were observed in lymphatic capillary density between healthy and diabetic or sedentary and trained groups. Data on blood vessels and their growth factors has been published previously (21).

Immunohistochemistry

Immunohistochemical staining of VEGF-D showed that in many of the diabetic muscles diffuse staining was found inside the muscle fibers that were swollen or infiltrated by macrophages, i.e., in damaged fibers (Fig. 3, C and D). Of the 10 studied diabetic muscles 2 contained >30 VEGF-D-positive fibers and the rest had between 1 and 10. This is <1% of the muscle fibers, because mouse MQF contains several thousand fibers (~8,000) in total. Damaged fibers were not seen in healthy muscles in the present study (Fig. 3D) or in our previous study (20). One diabetic mouse had an unusually large number of damaged fibers that were positive for VEGF-D (Fig. 3E). This mouse also had quantitatively one of the highest VEGF-D mRNA expressions. In addition to diffuse staining of VEGF-D in the swollen fibers, necrotic fibers with a large number of infiltrated macrophages stained intensively for VEGF-D (Fig. 3G). Larger vessels in the perimysium were also stained intensively for VEGF-D (Fig. 3H), but this staining was similar in healthy and diabetic mice. The staining pattern or intensity of VEGF-C and VEGFR-3 did not differ between healthy and diabetic or sedentary and exercise-trained mice.

VEGF-D Protein

VEGF-D protein concentration in serum was above the detection level of the ELISA kit in one-third of the samples, and there was no significant difference between healthy and diabetic mice in the serum VEGF-D values. No statistical comparison could be performed between each time point and group because of the low sample number with detected VEGF-D levels. VEGF-D concentration in the skeletal muscle homogenates was greater than in serum and above the detection level in all samples. Exercise training or diabetes did not affect the VEGF-D protein concentration significantly in muscle homogenates (Table 2).

DISCUSSION

To the best of our knowledge, this is the first study to report the effects of exercise on lymphatic vessels and lymphangiogenic growth factors in skeletal muscle. In addition, data about lymphatic vessel density in skeletal muscle have not been presented previously. The lymphatic part of the circulation in skeletal muscles has not been extensively studied, and most of our knowledge about lymphangiogenesis and lymphatic growth factors to date originates from cancer research. Lymphatic vessels have vital functions in the body. For instance, VEGF-C-deficient mice die before birth because of edema caused by the lack of lymphatic vessels (19). Earlier studies based on electron and light microscopy suggested that the lymphatic system in skeletal muscles consists only of larger lymphatic capillaries next to arterioles and veins (29). We recently showed (20) with specific antibodies for lymphatic endothelium that small lymphatic capillaries exist next to blood capillaries also in the capillary bed of skeletal muscles. However, the number of lymphatic capillaries in skeletal muscles is much lower than the number of blood capillaries.

In the present experiments we showed that in mouse skeletal muscle the expression of the two main lymphangiogenic growth factors, VEGF-C and VEGF-D, and their receptor VEGFR-3 is not affected by an acute running exercise bout or exercise training for 1, 3, or 5 wk. The only exception was found after acute running exercise, where VEGF-C mRNA expression was slightly increased 3 and 6 h after the exercise in healthy exercised muscle compared with control. All diabetic groups also had higher VEGF-C mRNA levels than the healthy control group. These findings are not supported, however, by the results from the exercise training experiment, where no differences were observed between healthy and diabetic groups or between sedentary and trained groups. Adaptation to exercise stimulus after training, which is known to attenuate mRNA responses, may explain this discrepancy.

Fig. 2. Effects of exercise training and diabetes for 1, 3, and 5 wk on the mRNA expression (normalized to GAPDH) of VEGF-C (A), VEGF-D (B), and VEGFR-3 (C) in skeletal muscle (experiment 2). Exercise training had no effect on any of the studied mRNAs. VEGF-D levels were increased significantly in diabetic skeletal muscles (B). The variation in the diabetic groups was large, because individual responses varied from 1.2- to 18.9-fold increase compared with the respective controls. VEGF-D expression was lower in exercised diabetic mice than in sedentary mice after 1 and 3 wk. **P < 0.01, *P < 0.05 vs. healthy control; #P < 0.05 vs. respective diabetic sedentary group (n = 5 in each group).
blood capillaries. arrows) were not distributed evenly surrounding all the muscle fibers like blood capillaries. scope shows thin vessel walls in lymphatic capillaries (arrows) locating next to capillaries with LYVE-1 (red).

Muscle blood perfusion may be increased up to 100-fold during was also similar in the healthy and diabetic groups. Skeletal in line with the growth factor expression results. Vessel density not change after exercise training in the present study, which is increased capillarization is a common phenomenon after en-

Also, samples from the training experiment were taken 24 h after the last training session to focus on the long-term rather than acute effects of exercise.

On the basis of the present findings, the responses of lymphatic vessels and growth factors to exercise seem to be different from the responses of blood vessels and angiogenic growth factors. Several studies have shown increased expression of VEGF-A and its receptors VEGFR-1 and VEGFR-2 after an acute exercise bout or training (6, 10–13, 23), and increased capillarization is a common phenomenon after endurance exercise training (16). Lymphatic vessel density did not change after exercise training in the present study, which is in line with the growth factor expression results. Vessel density was also similar in the healthy and diabetic groups. Skeletal muscle blood perfusion may be increased up to 100-fold during exercise (27), whereas lymph flow has been shown to be elevated ~5-fold at the onset of exercise and then decline to 2- to 3-fold compared with rest when exercise is continued (14, 22). It seems that the lymphatic part of the circulation, unlike blood vessels, is not challenged to its limits during exercise, and this could, at least partly, explain the observed differences in the growth factor responses. Considering the structure of the thin lymphatic vessel wall and the present results on their number and growth factor responses in skeletal muscle, we suggest that lymphatic vessels may enlarge rather than proliferate in response to exercise stimulus.

The present finding that exercise does not induce lymphangiogenesis cannot be explained by the exercise training protocol used, because it was shown to increase citrate synthase activity, which is a commonly used marker of endurance exercise training. In addition, our earlier study on angiogenesis responses from the same mice (21) showed that blood vessel density and growth factors were affected by both exercise and diabetes.

In adult human tissues, VEGF-D mRNA is most abundant in the heart, skeletal muscle, lungs, colon, and small intestine (1), suggesting that VEGF-D also has a role in skeletal muscle physiology, although yet largely unknown. In contrast to VEGF-C-deficient mice, VEGF-D-deficient mice are viable and have normal body weight and no defects in lymphatic function (5), indicating that VEGF-C is more important than VEGF-D for the development of the lymphatic system during embryonic development. Baldwin et al. (5) have suggested that in adult tissues the biological functions of VEGF-D could be more likely to be revealed in response to diseases or tissue damage. In both present experiments, a significant increase in VEGF-D mRNA expression was observed in the diabetic skeletal muscle. There was large variation in VEGF-D mRNA expression in the diabetic groups, because one-third of the diabetic mice had high expression, the majority had moderately increased values compared with healthy mice, and a few mice even had expression similar to healthy mice. Immunohistochemical analyses showed increased VEGF-D staining in some of the muscle fibers in diabetic muscles. Closer examination revealed that fibers that were stained for VEGF-D were damaged (i.e., swollen, necrotic, or containing centralized nuclei).

Table 2. VEGF-D protein concentration in skeletal muscle homogenates from experiment 2 measured with ELISA

<table>
<thead>
<tr>
<th>Group</th>
<th>VEGF-D Protein, pg/mg Total Protein</th>
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<tbody>
<tr>
<td>C1</td>
<td>147.4 22.7</td>
</tr>
<tr>
<td>C3</td>
<td>136.2 44.2</td>
</tr>
<tr>
<td>C5</td>
<td>131.9 22.7</td>
</tr>
<tr>
<td>T1</td>
<td>123.2 24.9</td>
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<tr>
<td>T3</td>
<td>150.3 71.5</td>
</tr>
<tr>
<td>T5</td>
<td>141.2 41.9</td>
</tr>
<tr>
<td>D1</td>
<td>143.7 66.3</td>
</tr>
<tr>
<td>D3</td>
<td>143.4 54.8</td>
</tr>
<tr>
<td>D5</td>
<td>112.1 10.9</td>
</tr>
<tr>
<td>DT1</td>
<td>153.6 36.3</td>
</tr>
<tr>
<td>DT3</td>
<td>140.9 44.4</td>
</tr>
<tr>
<td>DT5</td>
<td>142.4 19.9</td>
</tr>
</tbody>
</table>

No significant differences were detected between respective control and experimental groups. See MATERIALS AND METHODS for group descriptions.
VEGF-D staining was similar in the vessel walls of healthy and diabetic mice. This finding of VEGF-D-positive muscle fibers in diabetic muscles could explain the significantly higher mRNA expression in diabetic muscle homogenates. However, this increase in diabetic muscles was not detected with ELISA analysis of VEGF-D protein concentration either in serum or in muscle homogenates. Because the increased staining was found only inside the degenerating muscle fibers, it is not surprising that the serum concentrations were not changed and for most mice remained under the detection level of the method. It is also possible that the sensitivity of the ELISA kit was not sufficient to detect changes from the whole muscle homogenates, because most of the diabetic mice had only a few damaged fibers. The fiber damage observed in the diabetic mice in the present study is similar to what had already been found in exercise-induced muscle damage a few decades ago (e.g., Ref. 33). We found damaged fibers in both sedentary and exercised diabetic mice, but none in the healthy mice. Thus the fiber damage is more likely due to the hyperglycemia or the lack of insulin than to the exercise protocol. Whether VEGF-D expression is related to muscle fiber degeneration and inflammation processes in general, or specifically when hyperglycemia and/or hypoinsulinemia are present, needs to be studied further. Interestingly, serum glucose concentration and VEGF-D mRNA expression correlated positively, suggesting that VEGF-D expression increases along the severity of diabetes. From the present findings it is not possible to conclude as to which one occurs first in the damaged diabetic muscle fibers, the damage or the increased expression of VEGF-D. It can be speculated that VEGF-D would be produced in response to muscle damage, because it was also found in regenerating (i.e., healing) fibers and it has been shown to function as a growth factor at least for endothelial cells (9, 30, 31). However, the positive correlation with blood glucose levels may indicate that VEGF-D is produced in response to hyperglycemia before muscle damage has occurred.

The presence of VEGF-D protein in normal human arteries (20, 26) indicates that VEGF-D has a role in the maintenance of vascular growth and homeostasis. On the basis of the present finding that VEGF-D is increased in hyperglycemic muscles and found in damaged muscle fibers, we suggest that VEGF-D may also have a role in muscle degeneration and related inflammation and healing processes. VEGF-D staining in endothelia, smooth muscle cells, and macrophages in our samples is similar to the observations by Roy et al. (25). They studied atherosclerotic lesions in the aortas of diabetic rabbits and found increased immunostaining of VEGF-D in the intima of diabetic aortas, which may also be related to inflammatory processes (25).

In conclusion, in the present study we showed that both acute exercise and exercise training have no or only minor effect on lymphatic vessel density and mRNA expression of lymphangiogenic VEGF-C, VEGF-D, and VEGFR-3. This is different from the response of VEGF-A to exercise, and it is possible that lymphatic vessels are enlarged rather than proliferated in response to exercise. This is the first study to report lymphatic vessel density quantitatively in skeletal muscles. As another novel finding, we observed an increased expression of VEGF-D in diabetic muscles that may be related to muscle fiber damage and infiltration of macrophages.

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REFERENCES
20. Kivela R, Silvennoinen M, Touvrà AM, Lehtti TM, Kainulainen H, Viikko V. Effects of experimental type 1 diabetes and exercise training on...


