The influence of physical training on the angiopoietin and VEGF-A systems in human skeletal muscle

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Gustafsson T, Rundqvist H, Norrbom J, Rullman E, Jansson E, Sundberg CJ. The influence of physical training on the angiopoietin and VEGF-A systems in human skeletal muscle. J Appl Physiol 103: 1012–1020, 2007. First published June 14, 2007; doi:10.1152/japplphysiol.01103.2006.—Eleven subjects performed one-legged exercise four times per week for 5 wk. The subjects exercised one leg for 45 min with restricted blood flow (R leg), followed by exercise with the other leg at the same absolute workload with unrestricted blood flow (UR leg). mRNA and protein expression were measured in biopsies from the vastus lateralis muscle obtained at rest before the training period, after 10 days, and after 5 wk of training, as well as 120 min after the first and last exercise bouts. Basal Ang-2 and Tie-1 mRNA levels increased in both legs with training. The Ang-2-to-Ang-1 ratio increased to a greater extent in the R leg. The changes in Ang-2 mRNA were followed by similar changes at the protein level. In the R leg, VEGF-A mRNA expression responded transiently after acute exercise both before and after the 5-wk training program. Over the course of the exercise workload, there was a concurrent increase in basal VEGF-A protein and VEGF-R-2 mRNA in the R leg. Ki-67 mRNA showed a greater increase in the R leg and the protein was localized to the endothelial cells. In summary, the increased translation of VEGF-A is suggested to be caused by the short mRNA burst induced by each exercise bout. The concurrent increase in the Ang-2-to-Ang-1 ratio and the VEGF-expression combined with the higher level of Ki-67 mRNA in the R leg indicate that changes in these systems are of importance also in nonpathological angiogenic condition such as voluntary exercise in humans. It further establish that hypoxia/ischemia-related metabolic perturbation is likely to be involved as stimuli in this process in human skeletal muscle.

gene expression; ischemia; angiogenesis; exercise

ANGIOGENESIS IS A COMPLEX process involving many cell types, signaling pathways, growth factors, and receptors (6, 13, 18, 29, 30, 36). Vascular endothelial growth factor (VEGF)-A is a rate-limiting factor in this process (6, 29, 30). The VEGF receptor (VEGFR)-2 is essential for most of the VEGF-mediated angiogenic actions (29, 30). VEGFR-1 is thought to be a decoy receptor (16, 29, 30), although gene-deletion models suggest that its function is also important in the regulation of angiogenesis (16). In skeletal muscle, angiogenesis occurs as part of the normal adaptation to endurance exercise training (13, 18, 32), and VEGF-A activation of the VEGFR-2 is crucial in this process because exercise-induced angiogenesis is abolished in skeletal muscle in which the receptor is inhibited (22, 26, 27).

The angiopoietins, Ang-1 and Ang-2, comprise another important family of growth factors in theangiogenic process. The angiopoietins modify the biological effects of VEGF-A (2, 17, 24, 39). Ang-1 acts as an agonist and Ang-2 as an antagonist of their common receptor, Tie-2. Ang-2 destabilizes endothelial cells, which facilitates the effects of VEGF-A on endothelial activation, whereas Ang-1 helps to stabilize and maintain blood vessels. Such coordinated action controls vascular remodeling and maturation (2, 17, 24, 39). The biological effects and possible endogenous ligands of the other Tie receptor, Tie-1, are largely unknown, although Tie-1 is known to interact physically with Tie-2 in endothelial cells (7, 25) In animals, target disruption of the Tie-1 receptor shows that this receptor is required for normal vascular development, vessel maintenance, and endothelial cell survival (34).

Despite growing evidence that the angiogenic outcome depends largely on the coordinated expression of the angiopoietins and VEGF-A, to our knowledge, only one study in humans has measured the combined exercise-induced changes in these factors (37). However, in that study, mRNA expression was quantified only in the resting steady-state condition before and after 6 wk of endurance training at a constant workload (37). A leveling off in the expression of angiogenic factors occurs throughout muscle adaptation (11, 15, 19, 31). One could thereby assume that an experimental protocol that measures the expression of these factors only in steady state may overlook important changes in gene expression that might “peak” in the adaptation phase in skeletal muscle. Identifying the relative changes in various angiogenic growth factors throughout the angiogenic adaptation process will help us better understand the regulation of the angiogenic process in human skeletal muscle.

The overall aim of this study was to quantify temporally changes in the angiopoietin and VEGF systems, together with changes in the expression of Ki-67, a marker for cellular proliferation, during a 5-wk exercise-training period. Two different exercise conditions were used, with and without restricted blood flow, because restricted blood flow induces angiogenesis to a greater extent over a training period (35). We report that exercise induces changes in the angiopoietin system that may shift the balance to a more permissive condition for endothelial cell activation. We found greater expression of VEGF-A, VEGF-R-2, and a higher Ang-2-to-Ang-1 mRNA ratio in the restricted-blood flow condition, where also Ki67 expression in endothelial cells was increased to a greater extent. Together, these data indicate that coherent changes in...
the angiopoietin and VEGF-A systems are induced in a non-pathological angiogenic condition, such as exercise training in humans.

METHODS

Subjects

Eleven healthy male subjects were included in the study. Their mean (range) age, height, and weight were 24 (20–27) yr, 181 (173–190) cm, and 75 (63–90) kg, respectively, and their mean (range) Maximal O2 uptake was 51 (43–64) ml·kg⁻¹·min⁻¹. Well-trained subjects (Maximal O2 uptake > 65 ml·kg⁻¹·min⁻¹) were excluded to maximize the subjects’ training responses. The study was approved by the Ethics Committee of Karolinska Institutet. Before the study, the experimental protocol was explained to all of the subjects, and informed consent was obtained.

Experimental Model

Exercise in the supine position was performed with the dynamic constant-load knee-extension exercise (45 min, 60 revolutions/min). Each voluntary contraction extended the leg from 70 to 150° knee angle using an electromodynamically loaded cycle ergometer. Flexion was performed passively using the ergometer flywheel momentum to reposition the leg for the next extension. A method first described by Eiken and Bjurstedt (3) was used to restrict blood flow during exercise. The subject was positioned supine in the opening of a large pressure chamber with both legs inside the chamber and with a pad strapped to the calf of one leg. The pad was connected, via a metallic bar, to a crank arm of an electrically braked cycle ergometer with locked flywheel, the center of rotation being at the level of the heart. The chamber opening was sealed off at the level of the crotch by a rubber diaphragm with holes, and self-sealing sleeves for the legs. Shoulder supports were used to prevent cranial displacement of the body as the chamber pressure was increased. For exercise under restricted blood flow, the chamber pressure acting on the exercising leg was elevated to 50 mmHg above atmospheric pressure. This has been shown to reduce leg blood flow during one-legged cycle exercise by 15–20% (35). Exercise under nonrestricted blood flow was performed using the same experimental arrangements but at normal atmospheric pressure. The subjects performed one-legged exercise four times per week during a 5-wk period. Subjects exercised one leg for 45 min under restricted blood flow (R) condition and then the other leg under normal, unrestricted (UR) condition. The subjects were randomized into two groups: one group exercised their right leg and the other group exercised their left leg in the R condition. Each subject was instructed to exercise at the highest tolerable workload for 45 min, taking into account that he must complete the entire 45 min session. After 10 min of rest, the subject performed the same exercise protocol with the other leg. The two legs developed the same power and performed the same amount of work in each session. The workload for each exercise bout was measured and the total workload performed for each week was calculated.

Resting muscle biopsies were obtained using the percutaneous needle biopsy technique from the vastus lateralis muscle of both legs on three occasions: before the first exercise bout, after 10 days of training, and after 5 wk of training; the latter two biopsies were obtained 24 h after the previous bout of exercise. Exercise biopsies were obtained 2 h after the first and last (i.e., after 5 wk of training) exercise bouts. All biopsy samples were frozen within 10–15 s in liquid nitrogen and stored at −80°C until further analysis.

RNA Extraction and Reverse Transcription

Total RNA was prepared by the acid phenol method and quantified spectrophotometrically by absorbance at 260 nm. The integrity of total RNA was controlled on a 1% agarose gel electrophoresis. Two micrograms of RNA were reverse transcribed by Superscript reverse transcriptase (Life Technologies, Stockholm, Sweden) using random hexamer primers (Roche Diagnostics, Mannheim, Germany) in a total volume of 20 μl.

Real-Time PCR to Quantify mRNA

Detection of mRNA was performed on an ABI-PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA). Primer Express version 1.0 (Perkin-Elmer Applied Biosystems) designed oligonucleotide primers and TaqMan probes (Table 1). The probes covered exon-exon boundaries to avoid amplification of genomic DNA. Tie-1, Ang-1, and Ki67 were ordered as gene assays on demand (Hs00178500_m1, Hs00919197_m1, Hs00606991_m1, Perkin-Elmer Applied Biosystems). Selected as endogenous control to correct for potential variations in RNA loading was 18S rRNA (4310893E, Perkin-Elmer Applied Biosystems). β-Actin increases in response to repeated exercise, especially during restricted blood flow, as indicated by an increased amount relative to 18S levels, and thereby 18S was used as housekeeping gene. All reactions were performed in 96-well MicroAmp Optical plates, using the ABI-PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems) equipped with a charge-coupled device camera, which detects the signals from the fluorogenic probes during PCR. Amplification mixtures contained 5 μl of the diluted sample cDNA, 2× TaqMan Universal PCR Master mix, 300 nM of each primer, and 200 nM of the probe in a final volume of 25 μl. The mix for 18S rRNA was prepared according to the manufacturer’s recommendation and run at a 1:2,000 dilution in separate wells. Thermal cycling conditions included 2 min at 50°C, 10 min at 95°C, and then 45 cycles each of 15 s at 95°C and 1 min at 65°C. For every gene, all samples were amplified simultaneously in duplicate in one assay. As run standards, dilution series for all mRNA were used. The threshold cycle was determined for both the experimental gene and the endogenous control gene, and it was used to calculate the relative expression based on the respective standard curve equation. The expression level was normalized to the endogenous control gene. To compare the changes in the ratio of Ang-1 to Ang-2, the samples for each factor were run in duplicate in one assay to avoid any differences that may occur in different assays. This also makes it possible to directly compare the ratio between the two factors.

Table 1. Oligonucleotide sequences for primer and probe pairs used in TaqMan analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>ACTGCGATCCAAAATGGAACC</td>
<td>GATGCTTGTAAGATGACTCTGATCTT</td>
<td>TGTTGGAACATCTTCGGAGTACCTGTA</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>GCCATCTGGATCCCTGAACCTACA</td>
<td>TGCAGGAAATAGGACTCTGATCTAT</td>
<td>TAACCCCATGCTCTCGGAACGTTGACT</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>TCTTCTGTGCTAATGCTGCTCAGCTC</td>
<td>TGGAAAGATCCCTGCTTTAGTT</td>
<td>ACCCGGATTTAGCCGGCAAT</td>
</tr>
<tr>
<td>Ang-2</td>
<td>TGACAGCAGAAGTTTGGCAGAAAC</td>
<td>TGTTGGAAGGGATGTTTCCA</td>
<td>TGGAAAGCAGAATTTAATACAAGCAGCAGA</td>
</tr>
<tr>
<td>Tie-2</td>
<td>GGGCGATCTACTTGAAGAAATAGA</td>
<td>AATGATAGGTTAGAAGTTGGT</td>
<td>AGCCCGGATGAGGAGTGCCTC</td>
</tr>
</tbody>
</table>

VEGF, vascular endothelial growth factor; VEGFR-1 and -2, VEGF receptor-1 and -2; Ang-2, angiopoietin-2; Tie-2, Ang-2 receptor. All oligonucleotide sequences are written in the 5’ to 3’ direction. VEGF 5’ label 6-carboxyfluorescein (6FAM) and 3’ label N-tetramethylrhodamine (TAMRA). VEGFR-1 and VEGFR-2 5’ label 6FAM and 3’ label Dark Quencher.
without having to include housekeeping genes. The coefficients of variation of cycle threshold values in duplicate samples were <0.1%, ensuring negligible differences in efficiency and RNA-loading.

Protein Expression

A portion of the skeletal muscle biopsy was homogenized in ice-cooled buffer (40 μl/mg wet muscle) containing 0.1 M potassium phosphate (pH 7.7), 0.05% bovine serum albumin (BSA), 20 μg/ml leupeptin, 50 μg/ml aprotinin, and 40 μg/ml phenylmethylsulfonyl. The homogenate was rotated for 60 min at 4°C and centrifuged at 15,000 g for 10 min at 4°C. One hundred microliters of diluted supernatant (1:10) was used to quantify the VEGF protein by sandwich enzyme-linked immunoassay (Quantikine R&D systems, Minneapolis, MN). All techniques and materials were performed according to the manufacturer’s protocols. Monoclonal antibodies against VEGF-A (recognizing VEGF121 and VEGF165) and Ang-2 were used as the immobilized antibodies in the respective analyses, and a horseradish peroxidase-coupled polyclonal antibody was used as the secondary antibody. The optical density was quantified on a microplate reader μQuant (Bio-Tek Instruments, Winooski, VT). All samples were assayed in duplicate.

Immunohistochemistry

Frozen biopsy samples were embedded in OCT (Tissue-Tek, Sakura Finetek, Zoeterwoude, The Netherlands) and kept frozen at −80°C until further analysis. Cross sections (8 μm) of biopsy samples were cut at −20°C, placed on glass slides and immediately put in 2% paraformaldehyde for 30 min for fixation in room temperature, followed by washing in phosphate-buffered saline (PBS) 3 × 3 min. Section were then incubated in 1% H2O2 for 15 min in room temperature and washed in PBS for 3 × 3 min. After incubation with PBS containing 3% BSA and 1% normal swine serum (Dako, Glostrup, Denmark) for 1 h at room temperature, a rabbit anti-human Ki-67 antibody (Novocastro, Newcastle, UK) diluted 1:1,000 in PBS with 1% BSA and 0.3% Triton-X was applied to the sections and incubated at 4°C overnight. Subsequently, the sections were incubated with a biotin-conjugated swine anti-rabbit secondary antibody (Dako) diluted 1:500 in PBS with 1% BSA for 40 min at room temperature. The sections were washed in PBS for 3 × 3 min and incubated for 30 min in 1% avidin-biotin solution (ABC kit, Dako) in room temperature. After washing in PBS, the sections were visualized with diaminobenzidine (DAB; Sigma-Aldrich, Steinheim, Germany) for 3 min. Endothelial cells were then stained with FITC-conjugated Ulex europeus agglutinin diluted 1:500 in PBS for 15 min in room temperature. As a negative control, the primary antibody was excluded from the protocol. The sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA).

Statistics

The mRNA data were analyzed using logarithmic-transformed ratios (target/endogenous control). A two-way ANOVA for repeated measures was used to evaluate the effects of training (before, 10 days, and 5 wk) in the two exercise conditions (UR and R) on basal mRNA and protein content. A three-way ANOVA for repeated measures was used to evaluate the mRNA response to a single bout of exercise (preexercise and 2 h postexercise), before and after the 5 wk of training (before and after 5 wk), in the two exercise conditions (UR and R). Differences were considered significant at \( P < 0.05 \). Planned comparison was used (i.e., post hoc test) to locate differences corresponding to significant interactions or when no interaction was found to locate differences corresponding to significant main effects in the ANOVA models. Interactions in the ANOVA and the symbols that
Results

Training Response

The average cumulative workload (4 bouts) for each leg during each of the 5 wk of training were 1,975, 2,461, 2,803, 3,157, and 3,350 W, respectively, corresponding to a 1.3-, 1.5-, 1.8-, and 2.0-fold increase in workload over the last 4 wk compared with the first week.

mRNA

Pretraining biopsies. None of the measured variables differed significantly between the two legs before the first exercise bout.

VEGF-A. VEGF-A mRNA expression was increased 2 h after a single bout of exercise in the R leg (P < 0.001; Fig. 1, top left) before and after the training program (no interaction between a single bout of exercise and training). VEGF-A mRNA was not increased after a single bout of exercise in the UR leg before training, but there was a tendency to an increase after training (interaction between a single bout of exercise and training, P = 0.1; Fig. 1). The basal level of VEGF-A mRNA was not changed after training in the R leg, but it was lower after 10 days in the UR leg (effect of training in the UR leg, P < 0.05; Fig. 2) and remained at this level at the end of the 5 wk of training (interaction between training and condition, P < 0.05; Fig. 2).

VEGFR-2. VEGFR-2 mRNA expression was increased 2 h after a single bout of exercise in the R leg before training, but not after 5 wk of training (interaction between a single bout of exercise and training, P = 0.1; Fig. 1, bottom left). VEGFR-2 mRNA did not increase after a single bout of exercise in the UR leg before training, but there was a tendency to an increase after training (interaction between a single bout of exercise and training, P = 0.1). The basal level of VEGFR-2 mRNA was higher after 10 days in the R leg (P < 0.05), and even higher (P = 0.05) after 5 wk of training (effect of training in the R leg, P < 0.01; Fig. 2, bottom right). Basal VEGFR-2 mRNA levels did not change with training in the UR leg (interaction between training and condition, P < 0.05; Fig. 2).

Ang-1. The Ang-1 mRNA expression level did not change in response to a single bout of exercise regardless of exercise condition either before or after training. The basal level of Ang-1 mRNA was lower after 10 days of training (effect of training, P < 0.05; Fig. 3, top left) independent of training condition (no interaction between training and condition).

![Fig. 2. Basal levels of VEGF-A, VEGFR-1, VEGFR-2, mRNA, and VEGF-A protein in human vastus lateralis muscle over 5 wk of training. Values are means ± SD for n = 11 subjects except VEGF-A protein (n = 8). Pre, preexercise. C × T, interaction between condition (C) and training (T). ‡Significant differences (P < 0.05) between the exercise conditions and between before exercise and 10 days. ‡‡Significant differences (P < 0.05) between the 2 exercise conditions and between 10 days and 5 wk.](http://jap.physiology.org/)
Ang-2. The Ang-2 mRNA expression level did not change in response to a single bout of exercise regardless of exercise condition either before or after training. The basal level of Ang-2 mRNA was higher after 10 days of training (effect of training, $P < 0.01$; Fig. 3, top right) and remained elevated after 5 wk of training, independent of training condition (no interaction between training and condition).

Ang-2-to-Ang-1 mRNA-ratio. The ratio between the basal Ang-2 and Ang-1 mRNA levels was higher after 10 days of training and to a greater extent in the R leg (interaction between training and condition, $P < 0.05$; Fig. 4, middle right).

Tie-1. Tie-1 mRNA expression level did not change in response to a single bout of exercise, regardless of exercise condition either before or after training. The basal level of Tie-1 mRNA increased between 10 days and 5 wk of training (effect of training, $P < 0.05$; Fig. 4, bottom left) independent of training condition (interaction term between training and condition, $P = 0.1$).

Tie-2. Tie-2 mRNA expression level did not change in response to a single bout of exercise, regardless of exercise condition either before or after training. (Fig. 3) The basal level of Tie-2 mRNA did not change throughout the training program in either leg (Fig. 4, bottom left).

Ki-67. The basal level of Ki67 mRNA was higher after 5 wk of training and to a greater extent in the R leg (interaction between training and condition, $P < 0.01$; effect of training in the UR leg, $P < 0.05$; Fig. 5, left).

Proteins

VEGF-A. The VEGF-A protein level did not change in response to a single bout of exercise, in either condition, before or after training. The basal level of VEGF-A protein was higher after 10 days ($P < 0.05$) in the R leg, and showed a trend toward a further increase ($P = 0.08$) after 5 wk (main effect of training, $P < 0.05$; Fig. 2, top right). In the UR leg, the basal VEGF-A protein level did not change over the course of the training-period (interaction between training and condition, $P < 0.05$; Fig. 2).

Ang-2. The protein concentration of Ang-2 did not change in response to a single bout of exercise, in either condition, before or after the training period. The basal Ang-2 protein level increased after 10 days and remained elevated after 5 wk of training (effect of training, $P < 0.01$; Fig. 4, middle left), independent of training condition (no interaction between training and condition).

Ki-67. DAB-staining with Ki-67 stained positive cells brown, and FITC-Ulex europeus agglutinin, an endothelial marker, stained cells with fluorescing green. Immunohistochemical evaluation of the sections showed that the majority of the Ki-67 positive nuclei were colocalized with Ulex europeus staining. However, some Ki-67-positive nuclei did not appear to colocalized with Ulex europeus staining (Fig. 5, right).

DISCUSSION

The integrated functions of the VEGF and the angiopoietin systems in angiogenesis have been highlighted in both animal models and humans (2, 17, 24, 39). In this study, exercise with both restricted and unrestricted blood flow increased the basal Ang-2 mRNA level, whereas the Ang-1 mRNA level decreased slightly but significantly. Ang-2 protein expression followed the mRNA changes. A rat study reported similar exercise-induced responses of Ang-1 and Ang-2 mRNA expression (23). The animal findings and our data suggest that...
exercise induces a permissive condition for endothelial cell stimulation. Our laboratory recently reported that aerobic training increased the Ang-1 mRNA expression in human muscle (37). In that study we used constant workload with ordinary two legged cycling without any blood flow restriction throughout a 6-wk training period in contrast with the protocol in this study. Those divergent findings may thus be explained by the differences in exercise model/protocol and support the biological effects of Ang-1 as maturation signal (2, 17, 24, 39). This is also suggested by the fact that in our previous observation the Ang-1 mRNA level increased more in the subjects who adapted most to exercise training (high responders) and thus had a lower relative training stimulus in the latter part of the six-wk training period (37). The mRNA expression of the angiopoietin receptor Tie-2 did not change with training, whereas the Tie-1 mRNA level increased. The function of this receptor is largely unknown, but its expression is higher and is associated with increased Ang-2 mRNA expression in several conditions with increased angiogenic activity (4, 7, 25). Thus, we cannot exclude the possibility that changes in the expression of this receptor influence the exercise-induced angiogenesis process in human skeletal muscle.

The greater increase in the Ki-67 mRNA level after exercise with restricted blood flow indicates a greater turnover of proliferating cells. The protein localization of Ki-67 suggests that the increase in Ki-67 mRNA expression reflects mainly proliferation of the endothelial cells, as previously reported by Jensen et al. (19). This is also consistent with earlier studies using the same experimental model, which showed a greater angiogenic response in the leg trained with restricted blood flow (8). In contrast to the report by Jensen et al., Ki-67 was not exclusively expressed in the endothelial cells. This is, however, not surprising since several other cell-types in the skeletal muscle, such as satellite cells, may proliferate in response to repetitive exercise bouts. To identify these cells was, however, out of the scope of the present study.

The training-induced changes in Ang-1 and Ang-2 mRNA did not differ between the leg trained with restricted blood flow and that trained with unrestricted blood flow. However, the Ang-2-to-Ang-1 mRNA ratio increased after 10 days of train-...
ing in the leg exercised with restricted blood flow and was higher compared with the leg with unrestricted blood flow. This change was accompanied by an increase in VEGF-A expression. The combination of increased Ang-2-to-Ang-1 mRNA ratio and increased VEGF expression is indicative of a marked proangiogenic condition (2, 17, 24) and might explain the greater Ki-67 expression observed in this leg. Moreover, the level of VEGFR-2 mRNA increased exclusively in the leg with restricted blood flow, which might be resulted from an increase of the number of endothelial cells. However, if the changes in mRNA levels are accompanied by similar changes at the protein levels, the selective increase in basal VEGFR-2 expression may reflect a greater number of this receptor per endothelial cell, in contrast to VEGFR-1, and thus indicate a potentially greater stimulating capacity of VEGF-A.

Although there was no measurable change in the basal VEGF-A mRNA level, VEGF-A mRNA expression increased in response to a single bout of exercise in the leg exercised with restricted blood flow both before and after the training program. Thus the increase in VEGF-A mRNA seems to be transient and to occur in response to each exercise bout. However, the basal protein level of VEGF-A increased in the leg exercised under restricted blood flow. This suggests that increased translation in association with VEGF-A mRNA after each exercise bout generates more protein. An alternative explanation is that exercise increases cap-dependent translation of VEGF-A, which has been reported in other conditions that stimulate VEGF-A (9). However, such translatory mechanisms are generally downregulated in skeletal muscle during and immediately following endurance exercise (10). Thus we propose that the transient increase in VEGF-A mRNA expression associated with each exercise bout is responsible for the increased basal VEGF-A protein level. It further demonstrates that one cannot exclude changes at the protein levels despite unchanged mRNA levels. This might also explain previous reports that basal VEGF-A mRNA levels do not change in human skeletal muscle in response to training despite documented adaptation including angiogenesis (31, 37).

The mechanisms behind exercise-induced angiogenesis have been debated over many decades. In our experimental setting, factors associated with higher metabolic stress, including hypoxia, are more likely stimuli of the changed gene expression than exercise-induced increases in blood flow and the resultant changes in shear stress and wall tension. With exercise under blood flow restriction, shear stress should be lower due to a 15–20% lower blood flow in combination with increased compensatory vasodilatation (3, 35). A previous study using the same experimental model found no difference in activation of the major hypoxic transcriptional activator of VEGF-A, hypoxia inducible factor-1 (HIF-1), between exercise bouts with restricted and unrestricted leg blood flow (1). Nevertheless, in that study and in the present one, a greater increase in VEGF-A mRNA level occurred in the leg exercised with restricted blood flow. An alternative mechanism to changes in oxygen tension per se is that metabolism-related factors may be involved. We have earlier shown that exercise-induced changes in VEGF-A are correlated to exercise-induced increase in lactate concentration in skeletal muscle (14). Others have shown that metabolites stimulate angiogenesis in vitro (20, 28) an effect that is inhibited by VEGF-A neutralizing antibodies (5). Interestingly, activation of 5'-AMP-activated kinase (AMPK) has recently been reported to stimulate VEGF-A gene expression. Exercise activates AMPK by increasing the AMP-to-ADP ratio (38). Because the AMP-to-ADP ratio and the concentrations of lactate and adenosine increase more during exercise with restricted blood flow than during exercise with unrestricted blood flow (35), presumably this is followed by a greater AMPK activation in that condition.

The expression pattern of VEGF-A in response to a single bout of exercise under the two leg blood flow conditions provides indirect support that changes in metabolic factors rather than blood flow account for the increase in angiopoietin and VEGF-A expression. After 5 wk of training, the gene expression of VEGF-A after a single bout of exercise with unrestricted blood flow was similar to that observed after the very first bout of exercise in the leg exercising with restricted blood flow. We designed our protocol so that each subject performed maximal exercise in each bout throughout the study and the workload used in the exercise with restricted leg blood flow served as a reference. Such protocol results in a moderate workload in the unrestricted leg during the first exercise bouts but with a gradual increase throughout the 5-wk training program along with the gradual increase in exercise capacity in the restricted leg. Presumably, the workload in the leg exer-
cised with unrestricted leg blood flow was high enough to activate gene expression in the end of the 5-wk training period. The VEGFR-2 expression may also be related to changes in metabolite concentrations, because an increase in VEGF-A protein stimulates VEGFR-2 expression (12, 29, 30). It is tempting to speculate that the observed concomitant increases in the basal VEGFR-2 mRNA level and the basal VEGF-A protein level depend on such regulation.

The regulatory mechanisms as well as the cell source underlying angiopoietin expression in skeletal muscle tissue are much less known compared with the VEGF-A system. To our knowledge, no report exists that especially has analyzed skeletal muscle cells, but it is well accepted that both endothelial cells and cardiomyocytes express angiopoietins. Moreover, the expression of Ang-1 and Ang-2 mRNA can either be induced or repressed by hypoxia in an HIF-1-dependent manner (21). Our data showing slightly but significantly decreased Ang-1 mRNA levels after 10 days of exercise training and increased Ang-2 mRNA levels are consistent with a hypoxia-induced decrease in Ang-1 mRNA level and increased Ang-2 level in cardiomyocytes (21). These data are also consistent with a report on ischemic myocardial cells (33). The mechanisms regulating Tie-1 are also unclear, although VEGF-A increases the expression of Tie-1. Regardless of the mechanisms, the increase in Tie-1 mRNA levels occurred in both legs, whereas VEGF-A protein increased only in the leg with restricted blood flow. It should however, be noted that a trend (VEGF-A protein increased only in the leg with restricted blood flow). It should however, be noted that a trend (P = 0.1) to a greater increase was observed in the leg exercised with restricted blood flow. Nevertheless, other regulatory mechanisms may as well be as important for Tie-1 expression in the present experimental setting.

In summary, in human skeletal muscle, repetitive bouts of endurance exercise alter the balance of the angiopoietin system towards a condition known to be permissive for endothelial cell activation. Increased basal level of VEGF-A protein is associated with mRNA transients, induced by each exercise bout. Ang-2-to-Ang-1 mRNA ratio, VEGF-A and VEGFR-2 mRNA increases more after 5 wk of training with restricted blood flow and so does expression of Ki-67 in endothelial cells in the skeletal muscle. This suggests, that in response to exercise, the angiopoietin and the VEGF-A systems are transcriptional activated in a temporal fashion known to stimulate angiogenesis and that hypoxia/ischemia-related metabolic perturbation is likely to be involved as stimuli in this process in human skeletal muscle.

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**REFERENCES**


