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ANGIOGENIC GROWTH FACTOR RESPONSES TO LONG-TERM TREADMILL EXERCISE IN MICE

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Abstract : We sought to determine whether VEGF and other angiogenic growth factors and their receptors might be subject to negative feedback regulation during two weeks of treadmill-exercise conditioning in inbred strains of mice. C57BL/6 mice exhibited greater VEGF mRNA and protein responses in gastrocnemius muscle to a single bout of treadmill exercise compared to BALB/c mice. The patterns of VEGF, VEGFR1, VEGFR2, Ang2 and Tie2 mRNA expression in gastrocnemius muscles of C57BL/6 mice during long-term exercise support the hypothesis that they may be subject to negative feedback regulation. The combination of expression patterns for growth factors and their receptors suggests that multiple layers of control mechanisms may exist to prevent angiogenesis following a single bout of exercise and to promote angiogenesis following long-term exercise.

Key words : skeletal muscle mouse strains VEGF VEGFR1 VEGFR2 angiopoietins Tie2 negative feedback regulation

INTRODUCTION

The vascular system adapts chronically to metabolic demands of tissues. According to a metabolic hypothesis, vessel growth or regression results from chronic imbalances between perfusion capabilities of blood vessels and metabolic requirements of tissues (1, 2). Angiogenesis in skeletal muscles can be induced by chronic increases in metabolic rate resulting from exercise (3–5), electrical stimulation of muscle contraction (6–9), or chronic exposure to a cold environment (10–12).

Vascular endothelial growth factor (VEGF) stimulates angiogenesis. Chronic electrical stimulation of skeletal muscle contraction increases VEGF expression and stimulates angiogenesis (8). With prolonged stimulation, the upregulation of VEGF is attenuated, presumably because an increase in capillarity (8, 13, 14) promotes oxygen delivery to the muscles, thereby creating a

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new balance between oxygen delivery and oxygen demand. These observations suggest that VEGF production could be subject to negative feedback regulation. This concept of negative feedback regulation is supported by studies in skeletal muscles of rats (15), rabbits (16), and humans (4).

VEGF binds to two closely related receptor tyrosine kinases found on endothelial cells, VEGFR1 (flt1) and VEGFR2 (KDR/flk1) (17-20). VEGFR2 mediates the angiogenic effects of VEGF (18, 19). Although VEGFR1 binds VEGF with higher affinity than does VEGFR2, VEGFR1 is weakly phosphorylated in vascular endothelial cells. VEGFR1 is thus thought to be a negative regulator of angiogenesis, either by acting as a decoy receptor that limits bioavailability of VEGF to VEGFR2, or by suppressing signaling pathways initiated by VEGFR2 activation (18–20).

The angiopoietins and their Tie2 receptor also play a critical role in angiogenesis (20, 21). Angiopoietin-1 (Angl), which is produced by pericytes and vascular smooth muscle cells, binds to Tie2 receptors on endothelial cells, leading to interactions between endothelial cells, pericytes, and extracellular matrix that promote quiescence and stabilization of the mature vasculature. Angiopoietin-2 (Ang2), which is produced primarily by vascular endothelial cells (24), does not induce phosphorylation when it binds to Tie2 and thus counteracts the effects of Ang1 (20–23).

In the present study, we seek to establish a model for studying negative feedback regulation of angiogenic factors and their receptors during exercise conditioning. The following questions will be addressed: Does treadmill exercise induce significant VEGF responses in inbred strains of mice? Is the time course of mRNA expression of VEGF, VEGFR1, VEGFR2, Angl, Ang2, and Tie2 during treadmill exercise conditioning consistent with a concept of negative feedback regulation ?

MATERIALS AND METHODS

Animal protocols

Male C57BL/6 and BALB/c mice were acclimatized to the Laboratory Animal Facilities at the University of Mississippi Medical Center for one week prior to the experiments. The animals were provided with standard rodent chow and water *ad libitum*. All animal procedures were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee in accordance with federal guidelines.

Mice were randomly assigned to control and exercise groups. Mice assigned to exercise groups were familiarized with a motorized rodent treadmill (Exer 3/6, Columbus Instruments, Columbus OH) prior to the start of the experiments. In all experiments, the animals were handled and exercised at approximately the same time each day. In our initial experiments, VEGF responses to treadmill exercise were compared in 6-7 week old C57BL/6 and BALB/c mice. This involved a single 1 hour bout of exercise at 18 m/min at a 10° incline. Age-matched, cage-confined mice of the same strain were used as control groups. In all subsequent studies, male C57BL/6 mice (6-7 weeks old) were exercised on a rodent treadmill (18 m/min, 10° incline, 1 h/day)

for 1, 4, and 14 days. Age-matched, cage-confined male C57BL/6 mice were used as control subjects.

Tissues were collected between 1-2 hours following the last bout of exercise. The mice were anesthetized by isoflurane inhalation. Gastrocnemius muscles were quickly excised, rinsed with ice-cold PBS, blotted dry, weighed, snap-frozen, and stored in liquid nitrogen for later analyses. Following muscle harvest, the rats were humanely euthanized by increasing the level of isoflurane inhalation until death. Death was ensured by removing the heart.

Real-time RT-PCR primers (24, 25) (Table 1) were purchased from Sigma-Genosys (http://www.sigmaaldrich.com/Brands/Sigma Genosys.html). TRI Reagent® (TR 118) was purchased from Molecular Research Center, Inc. (Cincinnati, OH). DNA-FREE RNA kit (R1013) was purchased from Zymo Research, Inc. (Orange, CA). Agarose (162-0133), Protein Assay Reagent (500-0006), iScript® cDNA synthesis kit (170-8891), id™ SYBR® Green Supermix (170-8882) were purchased from BioRad Laboratories, Inc. (Hercules, CA). NE-PER® cytoplasmic and nuclear protein extraction kit (78833) and HALT® Protease Inhibitor Cocktail (78410) were purchased from Pierce Biotechnology, Inc. (Rockford, IL). Quantikine® colorimetric sandwich ELISA kit for mouse VEGF (MMVOO) was purchased from R&D Systems, Inc. (Minneapolis, MN). All other reagents were made from standard laboratory chemicals purchased from Sigma-Aldrich (St. Louis, MO).

RNA isolation

Frozen muscle was pulverized in a mortar and pestle under liquid nitrogen. The powdered tissue was added to TRI Reagent (Molecular Research Center, Inc.) in a 50 ml conical centrifuge tube, homogenized using a Polytron homogenizer on low speed. The sample was then transferred to a 15 ml conical centrifuge tube, centrifuged at 12,000 x g for 10 min at 4°C to remove large molecular weight DNA and insoluble structural proteins. The supernatant was transferred to a new 15 ml capped centrifuge tube and then processed in accordance with the manufacturer's protocol for RNA isolation. Aliquots of 5 μg of RNA isolate were treated with DNase I (DNA-free RNA kit, Zymo Research). RNA quantity and quality of each sample was assessed before and after DNase treatment. The concentration of nucleic acid was assessed before and after DNase treatment using a UV spectrophotometer (SmartSpec™ 3000, BioRad Laboratories). RNA quality was assessed by A260/A280 ratio and by electrophoresis of 0.9-1.0 μg aliquot on a 1.2% agarose gel using IX TBE buffer (26), with ethidium bromide staining. RNA was judged to be intact if the sample lane showed prominent discrete bands for 18S and 28S rRNA with no smearing.

cDNA synthesis

DNase-treated RNA (0.75 μg) from each sample was then used as a template for "+RT" reactions (i.e., cDNA synthesis) following the manufacturer's protocol (iScript® cDNA synthesis kit, BioRad Laboratories). "(–)RT" reactions, containing equivalent quantities of all the components of cDNA synthesis reaction, without reverse transcriptase (RT) enzyme, were produced in parallel to cDNA synthesis reactions. The cDNA samples were then diluted 1:10 with nuclease-free water

because preliminary studies (unpublished) indicated that this dilution produced the optimum range of fluorescence in the real-time RT-PCR reactions.

Real-time RT-PCR

Real-time RT-PCR assays were performed using iQ™ SYBR® Green Supermix (BioRad Laboratories) on an iCycler iQ™ Real-Time PCR Detection System (BioRad Laboratories). Undiluted (–)RT products were used as templates in negative control reactions to rule out the presence of genomic DNA contamination within the cDNA samples. Other negative control reactions included NT (no template) reactions (which contained SYBR® Green Supermix, forward and reverse primers, and nuclease-free water), as well as a blank (which contained only SYBR® Green Supermix and nuclease-free water). In experiments that involved more samples than could fit on one 96-well PCR plate, samples were divided evenly amongst 2 or more plates, with representative samples from each experimental group on each PCR plate. In these experiments, all PCR plates contained an extra positive control reaction (or "calibration sample") of rat skeletal muscle cDNA with 18S rRNA primers.

The average C_t for the target gene and the average C_t for 18S rRNA from the control group samples for each time point were used as a "reference sample". Relative Expression Ratios (27) were calculated using the following formula: Relative Expression Ratio = [Target Amplification Efficiency$^{(\text{Target}\Delta\text{Ct})}$]/ [18SrRNA Amplification Efficiency $^{<\text{18SrRNA } \Delta\text{ct})}$]; where Target ΔCt = target Ct for reference sample – average target C_t for sample, and 18SrRNA ΔCt – 18SrRNA C_t for reference sample - average 18SrRNA C_t for sample. The amplification efficiency for each set of primers was determined empirically using a dilution series of a pooled sample of mouse skeletal muscle cDNA.

Protein assays

Frozen gastrocnemius muscle was pulverized in a mortar and pestle under liquid nitrogen. The powdered tissue was added to Buffer I from NE-PER™ protein extraction kit (Pierce Biotechnology) containing HALT™ protease inhibitor cocktail (Pierce Biotechnology), following the manufacturer's protocol. Total protein in the protein isolates was quantitated using the microtiter plate protocol for BioRad Protein Assay (BioRad Laboratories). Protein samples were assayed using a mouse VEGFA ELISA kit (R&D Systems), following the manufacturers' protocol. The VEGFA protein concentration (pg/mL) for each sample was normalized by the total protein concentration (mg protein/mL), yielding pg VEGF/mg total protein.

Statistical analyses

InStat v. 3.0 Instant Biostatistics (GraphPad software) was used for all statistical analyses. This software tested all data sets by default for conformation to a normal distribution. Student's t-test for unpaired samples was used to compare treadmill-exercise vs. cage-confined groups for each mouse strain (experiment 1) or for each time point (experiment 2). Statistical significance was set at $P<0.05$. Data are reported as mean±standard error. Data expressed as "% change" were calculated for individual samples using the following

formula : % change = ((Value$_{experimental}$ – Value$_{control}$)/Value$_{control}$) × 100.

RESULTS

VEGF responses to treadmill exercise

A single 1 hour bout of treadmill exercise (18 m/min, 10° incline) significantly increased VEGF mRNA and protein levels in gastrocnemius muscle of C57BL/6 mice by 42±17% and 47±11%, respectively, compared to a cage-confined, age-matched control group (Fig. 1). In contrast, an identical bout of treadmill exercise did not have a significant effect on VEGF mRNA or protein expression in BALB/c mice compared to its control group (Fig. 1). Therefore, C57BL/6 mice were used for all subsequent experiments.

Time course of growth factor expression during exercise conditioning

Treadmill exercise significantly increased VEGF mRNA expression in gastrocnemius muscles of C57BL/6 mice on days 1 and 4 (42%±17 and 36%±16, respectively), but by

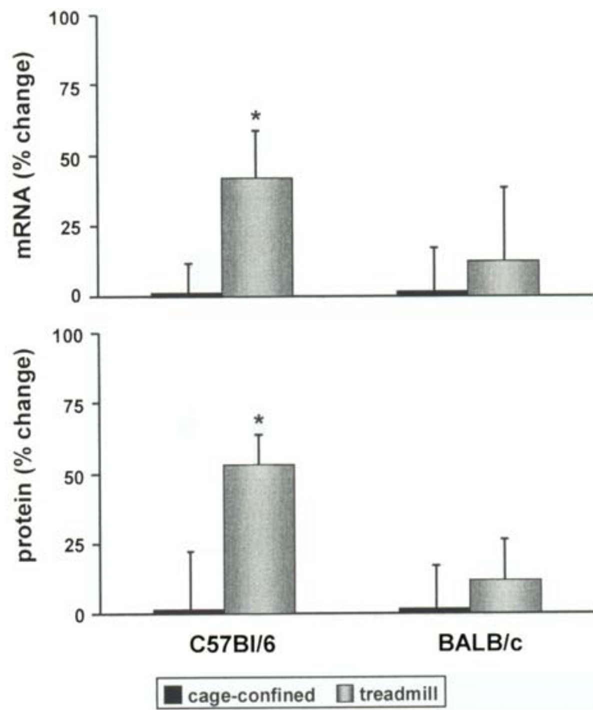

Fig. 1 : VEGF responses to a single bout of treadmill exercise. Male mice run on a motorized treadmill (18 m/min, 10° incline, 1 h) were compared to age-matched, cage-confined (control) mice of the same strain. Top panel: VEGF mRNA expression (normalized by 18S rRNA expression) in gastrocnemius muscle, assayed by real-time RT-PCR. Bottom panel: VEGF protein (normalized by total soluble protein) from gastrocnemius muscle, assayed by ELISA. Data are expressed as Mean±SE. *P<0.05 vs. control. C57BL/6 control, n=6; C57BL/6 exercise, n=6; BALB/c control, n=5; BALB/c exercise, n=4.

TABLE I : Real-time RT-PCR primers.

Target	*Genebank accession#*		*Primer sequence*	*Amplicon length (bp)*
VEGFA	NM_009505	fwd	5'-CACGACAGAAGGAGAGCAGAAG-3'	147
		rev	5'-ACACAGGACGGCTTGAAGTG-3'	
VEGFR1	NM_010228	fwd	5'-GTCGGCTGCAGTGTGTAAGT-3'	65
		rev	5'-TGCTGTTCTCATCCGTTTCT-3'	
VEGFR2	NM_010612	fwd	5'-TGTCAAGTGGCGGTAAAGG-3'	89
		rev	5'-CACAAAGCTAAAATACTGAGGACTTG-3'	
Ang1	NM_009640	fwd	5'-CTACCAACAACAACAGCATCC-3'	105
		rev	5'-CTCCCTTTAGCAAAACACCTTC-3'	
Ang2	NM_007426	fwd	5'-CTGTGCGGAAATCTTCAAGTC-3'	146
		rev	5'-TGCCATCTTCTCGGTGTTG-3'	
Tie2	E08401	fwd	5'-CGGCCAGGTACATAGGAGGA-3'	86
		rev	5'-CCCCCACTTCTGAGTTCAC-3'	
18S rRNA	X00686	fwd	5'-CGGCGACGACCCATTCGAAC-3'	99
		rev	5'-GAATCGAACCCTGATTCCCCGTC-3'	

day 14, VEGF mRNA was not significantly different from the age-matched, cage-confined control group (Fig. 2, top panel). Treadmill exercise significantly increased VEGFR1 mRNA expression in gastrocnemius muscles at all time points examined; however, the level of expression was attenuated during the two weeks of exercise training. For example, VEGFR1 mRNA expression was increased by 69%±28 on day 1, 47%±18 on day 4, and 27%±17 on day 14, compared to age-matched, cage-confined controls (Fig. 2, center panel). VEGFR2 mRNA expression in gastrocnemius muscles decreased in response to treadmill exercise on days 1 and 4 (–45%±6 and –26%±7, respectively), and returned to near basal levels by day 14 (Fig. 2, bottom panel).

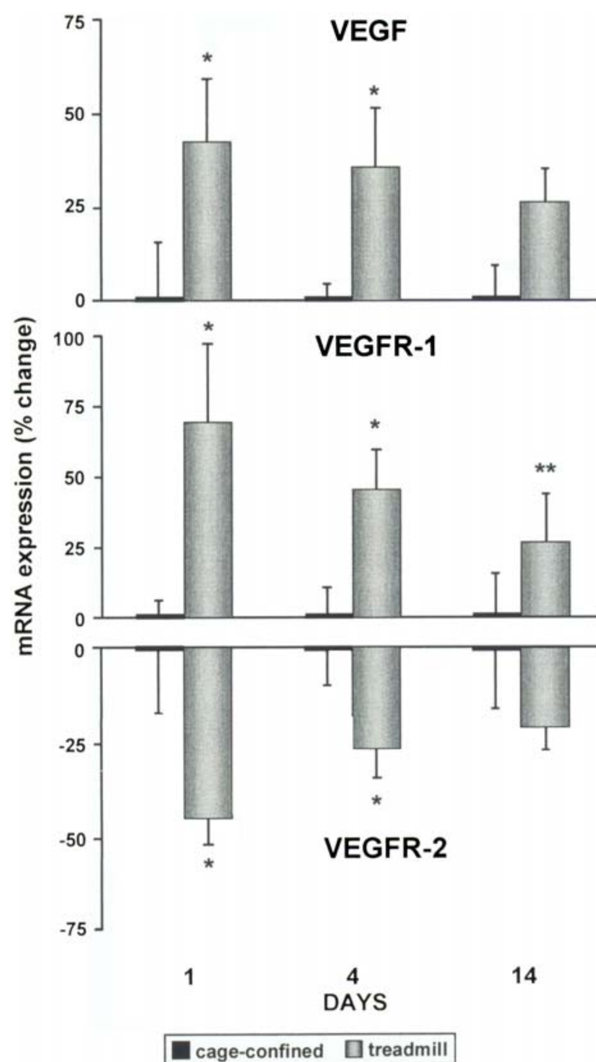

Fig. 2 : Temporal patterns of mRNA expression for VEGF and its receptors during treadmill-exercise conditioning. Male C57BL/6 mice run on a motorized treadmill (18 m/min, 10° incline for 1 h/day) were compared to age-matched, cage-confined (control) mice. Target mRNA expression (normalized by 18S rRNA expression) in gastrocnemius muscle was assayed by real-time RT-PCR. Data are expressed as Mean±SE. *P<0.05 vs. control, **P<0.01 vs. control. Day 1: control, n=6; exercise n=6. Day 4: control, n=7; exercise, n=7. Day 14: control, n=8; exercise, n=7.

Treadmill exercise increased Ang2 mRNA expression in gastrocnemius muscles by 47%±23 on day 1, and returned to basal levels by day 4 (Fig. 3, center panel). Exercise significantly decreased Tie2 mRNA expression by 27%±10 on day 1, but then returned to near basal levels by day 4 (Fig. 3, bottom panel). Angl mRNA expression was not affected by treadmill exercise at any of the time points examined (Fig. 3, top panel).

DISCUSSION

Angiogenic growth factor responses to treadmill exercise were examined herein at three critical time points in C57BL/6 mice: (a) day 1, after the first bout of exercise; (b) day 4, when VEGF levels would be expected to be high, but capillarity in muscles would not have sufficient time to increase significantly; and, (c) day 14, after angiogenic adaptation to chronic exercise would be significant (1, 8, 15, 28). Exercise caused an initial increase in VEGF, VEGFR1, and Ang2 mRNA levels; and, an initial decrease in VEGFR2 and Tie2 mRNA levels, which was followed two weeks later by a return to nearly basal levels. The results

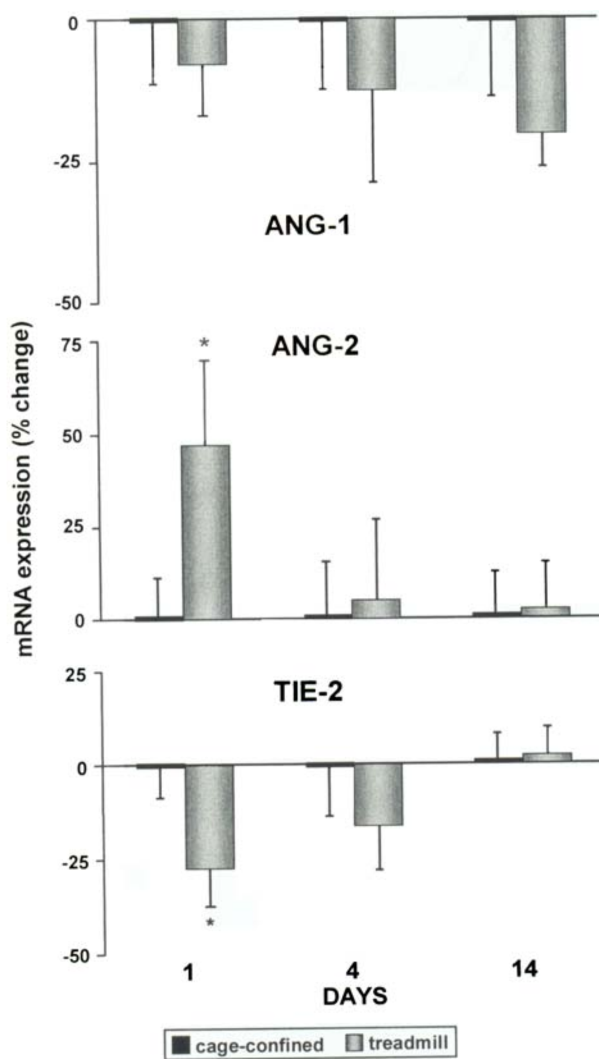

Fig. 3 : Temporal patterns of mRNA expression for angiopoietins and Tie2 during treadmill-exercise conditioning. Male C57BL/6 mice run on a motorized treadmill (18 m/min, 10° incline, 1 h/day) were compared to age-matched, cage-confined (control) mice. Target mRNA expression (normalized by 18S rRNA expression) in gastrocnemius muscle was assayed by real-time RT-PCR. Data are expressed as Mean±SE. *$P<0.05$ vs. control. Day 1: control, n=6; exercise, n=6. Day 4: control, n=7; exercise, n=7. Day 14: control, n=8; exercise, n=7.

therefore support the hypothesis that VEGF, VEGFR1, VEGFR2, Ang2, and Tie2 could be subject to negative feedback regulation during treadmill exercise in C57BL/6 mice. C57BL/6 mice exhibited a greater VEGF response to treadmill exercise than did BALB/c mice (Fig. 1). Other studies show that BALB/c mice have a lower density of collateral vessels, reduced angiogenesis during ischemia, and a reduced production of VEGF compared to C57BL/6 mice (29, 30). Our studies thus confirm that BALB/c mice have an impaired VEGF response to exercise, compared to C57BL/6 mice.

Normally, a single bout of exercise does not lead to significant angiogenic activity; rather, a series of repeated bouts of exercise over a period of days to weeks is required to increase capillarity significantly in skeletal muscles (15, 28). The mRNA expression patterns observed for VEGF receptors (Fig. 2) could explain these latter findings. For example, decreased production of VEGFR2 could serve to decrease endothelial cell sensitivity to the VEGF stimulus, and, a simultaneous increase in production of high affinity VEGFR1 could serve to decrease VEGF availability to the VEGFR2 receptor following a single bout of exercise. Thus, the contrasting mRNA responses of VEGFR1 and VEGFR2 observed here could have an important *in vivo* function to prevent initiation of angiogenesis in response to a single, spurious, transient increase in local VEGF levels.

The level of angiogenic activity in virtually any tissue depends upon a balance between stimulatory and inhibitory influences of growth factors. Ang2 competes with Angl for binding sites on Tie2 receptors. When Tie2 binding of Ang2 predominates, vascular destabilization occurs and angiogenesis follows. The results of the present study showing that a single bout of

exercise can increase Ang2 expression, decrease Tie2 expression, and have no effect on Angl expression suggests multilayered mechanisms aimed to destabilize existing vasculature in preparation for subsequent angiogenesis. At the same time, the exercise-induced increase in VEGFR1 expression and simultaneous decrease in VEGFR2 expression would act to attenuate or halt angiogenic activity following a single, spurious bout of exercise. Therefore, the various changes in growth factor/receptor expression patterns observed here would seem to ensure that increases in capillarity do not inappropriately exceed the level needed to match the oxygen delivery capacity of the vasculature to increased metabolic demands of the exercising muscles.

In conclusion, we demonstrate in the current study that a single bout of treadmill exercise caused a greater VEGF response in C57BL/6 mice compared to BALB/c mice. The temporal patterns of mRNA expression of VEGF, VEGFR1, VEGFR2, Ang2, and Tie2 support the hypothesis that these angiogenic growth factors may be subject to negative feedback regulation in exercising skeletal muscle of C57BL/6 mice. The expression patterns also suggest the existence of multiple levels of control mechanisms that tightly regulate exercise-induced angiogenesis.

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REFERENCES

1. Adair, TH, Gay WJ, Montani J-P. Growth regulation of the vascular system: evidence for a metabolic hypothesis. *Am J Physiol Regulatory Integrative Comp Physiol* 1990; 259: R393–R404.
2. Semenza GL. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. *Physiology* (Bethesda) 2009; 24: 97–106.
3. Egginton, S. Invited review: activity-induced angiogenesis. *Pflugers Arch* 2009; 457: 963–977.
4. Richardson RS, Wagner H, Mudaliar SR et al. Exercise adaptation attenuates VEGF gene expression in human skeletal muscle. *Am J Physiol Heart Ore Physiol* 2000; 279: H772–H778.
5. Suzuki J, Gao M, Batra S et al. Effects of treadmill training on the arteriolar and venular portions of capillary in soleus muscle of young and middle-aged rats. *Ada Physiol Scand* 1997; 159: 113–121.
6. Adair TH, Hang J, Wells ML et al. Long-term electrical stimulation of rabbit skeletal muscle increases growth of paired arteries and veins. Am *J Physiol Heart Circ Physiol* 1995; 269: H717–H724.
7. Hang J, Fleming JB, Wells ML et al. Miniaturized electrical stimulator with controllable duty cycles. *Am J Physiol Heart Circ Physiol* 1995; 268: H1373–H1378.
8. Hang J, Kong L, Gu J-W et al. VEGF gene expression is upregulated in electrically stimulated rat skeletal muscle. *Am J Physiol Heart Circ Physiol* 1995; 269: H1827–H1831.
9. Hudlicka O, Dodd L, Renkin EM et al. Early changes in fiber profile and capillary density in long-term stimulated muscles. *Am J Physiol Heart Circ Physiol* 1982 243: H528–H535.
10. Banchero N., Kayar SR, AJ. Lechner AJ. Increased capillarity in skeletal muscle of growing guinea pigs acclimated to cold and hypoxia. *Respir Physiol* 1985; 62: 245–255.
11. Sillau AH, Aquin L, Lechner AJ et al. Increased

capillary supply in skeletal muscle of guinea pigs acclimation to cold. *Respir Physiol* 1980; 42: 233–425.

12. Suzuki J, Gao M, Ohinata H et al. Chronic cold exposure stimulates microvascular remodeling preferentially in oxidative muscles in rats. *Jap J Physiol* 1997; 47: 513–520.
13. Dawson JM, Hudlicka 0. The effect of long-term activity on the microvasculature of rat glycolytic skeletal muscle. *Int J Microcirc Clin Exp* 1989; 8: 53–69.
14. Hudlicka O, Price S. The role of blood flow and/ or muscle hypoxia in capillary growth in chronically stimulated fast muscles. *Pflügers Arch* 1990; 417: 67–72.
15. Lloyd PG, Prior BM, Yang HT et al. Angiogenic growth factor expression in rat skeletal muscle in response to exercise training. *Am J Physiol Heart Cirm Physiol* 2003; 284: H1668–H1678.
16. Skorjanc D, Jaschinski F, Heine G et al. Sequential increases in capillarization and mitochondrial enzymes in low-frequency-stimulated rabbit muscle. *Am J Physiol Cell Physiol* 1998; 274: C810–C818.
17. Cao Y. Positive and negative modulation of angiogenesis by VEGFR1 ligands. *Sci Signal* 2009; 2(59): re1.
18. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocrine Rev* 2004; 25: 581–611.
19. Olsson A-K, Dimberg A, Kreuger J et al. VEGF receptor signaling – in control of vascular function. *Nat Rev Mol Cell Biol* 2006; 7: 359–371.
20. Yancopoulos GD, Davis S, Gale NW et al. Vascular-specific growth factors and blood vessel formation. *Nature* 2000; 407: 242–248.
21. Holash J, Wiegand SJ, Yancopoulos GD. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene* 1999; 18: 5356–5362.
22. Imhof BA, Aurrand-Lions M. Angiogenesis and inflammation face off. *Nat Med* 2006; 12: 171–172.
23. Suri C, Jones PF, Patan S et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 1996; 87: 1171–1180.
24. Gerber HP, Kowalski J, Sherman D, et al. Complete inhibition of rhabdomyosarcoma xenograft growth and neovascularization requires blockade of both tumor and host vascular endothelial growth factor. *Cancer Res* 2000; 60: 6253–6258.
25. Kelly BD, Hackett SF, Hirota K et al. Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. *Circ Res* 2003; 93: 1074–1081.
26. Sambrook J, Fitsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, Cold Spring Harbor Press 1989; appendix B, p.23.
27. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; 29: e45.
28. Prior BM, Yang YT, Terjung RL. What makes blood vessels grow with exercise? *J Appl Physiol* 2004; 97: 1119–1128.
29. Zbinden S, Clavijo LC, Kantor B et al. Interanimal variability in preexisting collaterals is a major factor determining outcome in experimental angiogenesis trials. Am *J Physiol Heart Circ Physiol* 2007; 292: H1891–H1897.
30. Chalothorn D, Clayton JA, Zhang H et al. Collateral density, remodeling and VEGF-A expression differ widely between mouse strains. *Physiol Genomics* 2007; 30: 179–191.