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## Angiogenic growth factor expression in rat skeletal muscle in response to exercise training

Pamela G. Lloyd,<sup>1</sup> Barry M. Prior,<sup>1</sup> Hsiao T. Yang,<sup>1</sup> and Ronald L. Terjung<sup>1,2,3</sup>

<sup>1</sup>Department of Biomedical Sciences, College of Veterinary Medicine, <sup>2</sup>Department of Physiology, College of Medicine, and <sup>3</sup>Dalton Cardiovascular Research Center, University of Missouri, Columbia, Missouri 65211

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**Lloyd, Pamela G., Barry M. Prior, Hsiao T. Yang, and Ronald L. Terjung.** Angiogenic growth factor expression in rat skeletal muscle in response to exercise training. *Am J Physiol Heart Circ Physiol* 284: H1668–H1678, 2003. First published January 23, 2003; 10.1152/ajpheart.00743.2002.—Angiogenesis occurs in skeletal muscle in response to exercise training. To gain insight into the regulation of this process, we evaluated the mRNA expression of factors implicated in angiogenesis over the course of a training program. We studied sedentary control ( $n = 17$ ) rats and both sedentary ( $n = 18$ ) and exercise-trained ( $n = 48$ ) rats with bilateral femoral artery ligation. Training consisted of treadmill exercise (4 times/day, 1–24 days). Basal mRNA expression in sedentary control muscle was inversely related to muscle vascularity. Angiogenesis was histologically evident in trained white gastrocnemius muscle by *day 12*. Training produced initial three- to sixfold increases in VEGF, VEGF receptors (KDR and Flt), the angiopoietin receptor (Tie-2), and endothelial nitric oxide synthase mRNA, which dissipated before the increase in capillarity, and a substantial (30- to 50-fold) but transient upregulation of monocyte chemoattractant protein 1 mRNA. These results emphasize the importance of early events in regulating angiogenesis. However, we observed a sustained elevation of the angiopoietin 2-to-angiopoietin 1 ratio, suggesting continued vascular destabilization. The response to exercise was (in general) tempered in high-oxidative muscles. These findings place importance on cellular events coupled to the onset of angiogenesis.

capillaries; peripheral vascular disease; endothelium; cytokines; chemokines

PATIENTS WITH PERIPHERAL VASCULAR DISEASE (PVD) experience pain on exertion (intermittent claudication) due to insufficient oxygen delivery to the exercising muscle. These symptoms could potentially be relieved by increasing blood flow to the muscle via remodeling of existing collateral arteries (arteriogenesis) and/or by increasing muscle capillarity (angiogenesis). Exercise training can stimulate both arteriogenesis and angiogenesis, as seen by the increased collateral-dependent blood flow (45) and greater muscle capillarity (15) after training. Thus exercise is a valuable treatment for PVD (8). However, many patients with PVD are unable or unwilling to exercise. Examination of the underlying mechanisms of arteriogenesis and angiogenesis in re-

sponse to exercise training could lead to improved treatments for these patients. Therefore, in this study, we set out to characterize the expression of a variety of angiogenic factors in skeletal muscle of rats with experimental PVD (femoral artery occlusion) over the course of a 24-day exercise training program. Our goals were to 1) identify factors involved in training-induced angiogenesis and 2) investigate how the responses of the individual growth factors are coordinated to produce the overall angiogenic response.

To achieve these goals, we first examined the expression of angiopoietin 1, angiopoietin 2, and their receptor Tie-2 over the course of the training program. The angiopoietins act in concert with VEGF (3) and are critically important for angiogenesis. Angiopoietin 1 stabilizes the existing vasculature (40), whereas angiopoietin 2 destabilizes it, allowing new growth to occur (26). Because both angiopoietins act through the same receptor (Tie-2), changes in the ratio of angiopoietin 2 to angiopoietin 1 have been implicated in angiogenesis, with a higher angiopoietin 2-to-angiopoietin 1 ratio being proangiogenic in the presence of VEGF (17). We also assessed changes in the abundance of monocyte chemoattractant protein (MCP)-1 mRNA in response to training. MCP-1 accelerates vascular remodeling in response to ischemia (16), suggesting that MCP-1 could be involved in skeletal muscle angiogenesis.

In addition to the angiopoietins and MCP-1, we also investigated the effect of the training program on VEGF, VEGF receptors, and endothelial nitric oxide (NO) synthase (eNOS). VEGF is increased immediately after a single exercise bout in both animals (4) and humans (14). Exercise also elevates mRNA for the VEGF receptor Flt (9), although the response of the KDR receptor remains unclear (11). Likewise, expression of eNOS is enhanced by exercise (12, 22, 24, 43). Although acute exercise has previously been shown to alter the expression of these factors, their pattern of expression during chronic exercise is not well characterized. Likewise, the relationship between changes in these factors and potential changes in angiopoietin expression during the course of a training program has not been explored.

Measurements of growth factor mRNA were made in muscle sections of contrasting vascularity (51), allow-

Address for reprint requests and other correspondence: R. L. Terjung, Dept. of Biomedical Sciences, E102 Veterinary Medicine Bldg., 1600 E. Rollins Rd., Univ. of Missouri, Columbia, MO 65211 (E-mail: TerjungR@missouri.edu).

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ing us to identify fiber-type differences in both basal and exercise-stimulated expression. We hypothesized that training would produce the greatest effect on growth factor expression in the sparsely vascularized white gastrocnemius (WG) muscle, because this is the tissue most at risk of ischemia during exercise in animals with femoral artery ligation. Our results show that significant alterations in angiotensin, Tie-2, MCP-1, eNOS, VEGF, and VEGF receptor mRNA occur during exercise training. These findings illustrate a pattern of response that differs among factors and depends on fiber type. Most of the factors increased initially, but declined before angiogenesis was histologically evident, whereas other factors showed a sustained increase throughout the course of the training program.

## MATERIALS AND METHODS

### *Study Design*

Rats were divided into three main experimental groups: sedentary controls (Cont;  $n = 17$ ), sedentary ligated (LIG;  $n = 18$ ), and exercise-trained ligated (LIG-EX;  $n = 48$ ).

### *Animal Care*

Eighty-three male Sprague-Dawley rats (~325 g, Taconic Farms; Germantown, NY) were used in this study. Rats were housed two per cage in temperature-controlled rooms (21°C) with a 12:12-h light-dark cycle. Rat chow and water were provided ad libitum. Before the study began, rats were familiarized with the treadmill by walking them at 15–20 m/min, 15% grade, for ~5 min. They were also taught to run at the front of the treadmill belt by turning the treadmill on and off. During the familiarization period, rats were placed on the treadmill two to three times per day for 3–5 days. This protocol does not induce peripheral adaptations in rats (44, 46, 51).

The experimental protocols used in this study were approved by the Animal Care and Use Committee of the University of Missouri and conform to National Institutes of Health guidelines.

### *Femoral Artery Ligation*

In LIG and LIG-EX rats, the femoral arteries were ligated bilaterally ~5–6 mm distal to the inguinal ligament. The rats were anesthetized with ketamine (100 mg/kg) and acepromazine (0.5 mg/kg). The skin of the inner thighs was shaved and swabbed with Betadine. Small incisions were made in the skin, and the right and left femoral arteries were isolated and completely occluded by ligation with 3-0 surgical silk (44, 49, 50). The wounds were closed, and the animals were allowed to recover in their cages. The rats were given a full day of recovery before beginning the exercise training program. After 1 day, all animals appeared fully recovered and showed no signs of tissue necrosis.

### *Exercise Training*

Rats in the LIG-EX group exercised daily on a motor-driven rodent treadmill (Quinton model 42-15) four times per day (once per hour in the morning). The rats began by walking at 15 m/min and progressed to running at 20–25 m/min as their ability to exercise increased. Rats exercised

until showing signs of fatigue (hopping gait). The daily exercise time of each rat was recorded.

To study the time course of angiogenic growth factor expression in response to training, rats followed the training program for 1, 3, 8, 12, 18, or 24 days (3 and 8 days,  $n = 12$ ; all other groups,  $n = 6$ ). Exercise was performed 7 days/wk except for *days 14* and *19*. On the last day of training, tissue was collected 2 h after the final exercise bout. Cont rats and LIG rats were euthanized at the same time points for comparison (Cont,  $n = 2$ –3/day; LIG,  $n = 2$ –4/day). However, fewer Cont animals than LIG-EX animals were taken per time point, because it seemed likely that cage activity would provide little angiogenic stimulus in normal animals over the 24 days of the experiment. Similarly, fewer LIG animals than LIG-EX animals were taken per time point, because even after femoral artery occlusion blood flow capacity to the calf muscles is much greater (~3-fold) than is needed for resting flow demands (51). Thus the calf muscles are not frankly ischemic in the absence of extensive muscle recruitment (such as occurs during treadmill exercise), and thus there was again expected to be little stimulus for angiogenesis in these animals.

### *Tissue Collection and Processing*

Under deep pentobarbital anesthesia (60 mg/kg ip), muscles were collected from both hindlimbs. The superficial white section of the medial head of the gastrocnemius (WG; primarily fast-twitch white fibers), the deep red section of the lateral gastrocnemius (RG; primarily fast-twitch red fibers), and the soleus (SOL) muscle (primarily slow-twitch red fibers) were resected, rapidly frozen with liquid N<sub>2</sub>-cooled aluminum tongs, and stored at –80°C. A ~5-mm cross section of the WG muscle was mounted on a cork, frozen in liquid N<sub>2</sub>-cooled isopentane, and stored at –80°C for histochemical analysis of capillarity.

For RNA isolation, ~50 mg of tissue were snapped off of each muscle under liquid nitrogen. The frozen muscle sample was placed in a plastic tube inside an aluminum mortar immersed in liquid nitrogen and powdered with a frozen aluminum pestle. The frozen powder was then transferred to a tube containing TRIzol (Life Technologies; Frederick, MD). Total RNA was isolated according to the manufacturer's instructions. Integrity of the RNA was assessed by separating ~2 µg of each sample on a 1% agarose gel containing ethidium bromide and examining the 5S, 18S, and 28S rRNA bands under ultraviolet light.

Total RNA was treated with DNase (DNA Free, Ambion, Austin, TX) to remove contaminating genomic DNA. The treated RNA was assayed by spectrophotometer (A260/A280) to assess purity and concentration. All samples were then diluted to 5 ng/µl with Tris-EDTA buffer (Sigma Chemical; St. Louis, MO). Samples were stored at –80°C.

### *Tissue Capillarity*

Angiogenesis was assessed by determining the increase in capillary contacts per fiber in the WG section. Ten-micrometer-thick frozen sections were cut using a cryostat (CM 1850, Leica). Capillaries were visualized by staining acetone-fixed tissue sections for alkaline phosphatase activity (35) and counterstaining with metanil yellow, as previously described (25, 47, 48, 51). Images of each muscle were collected with a Spot digital camera (Diagnostic Instruments; Sterling Heights, MI) connected to a Nikon Eclipse E600 microscope (Nikon; Torrance, CA). For each muscle, a composite image of the entire section was created using Adobe Photoshop software. This image was overlaid with a 20-square grid, allow-

ing 20 nonoverlapping fields ( $\sim 0.06 \text{ mm}^2$  each) to be identified for analysis. A new image was then acquired in each selected field. The number of myocytes and number of capillaries surrounding each myocyte were counted using Photoshop software and recorded for calculation of capillary contacts per fiber. A total of  $\sim 130$  fibers was examined for each muscle.

#### Real-Time Quantitative RT-PCR

Expression of angiogenic mRNAs was studied by RT-PCR. TaqMan RT-PCR reagents, obtained from Applied Biosystems (Foster City, CA), were used according to the manufacturer's protocols (TaqMan Universal PCR Master Mix Protocol No. 4304449, Revision A). Total RNA (50 ng) was converted to cDNA by RT primed by random hexamers. Specific primers and fluorescent probes were used for each target. Primers and probes were designed using Primer Express (Applied Biosystems). The sequences were as follows: VEGF, TTC-AAGCCGTCCTGTGTGC (forward), TCCAGGGCTTCAT-CATTGC (reverse), FAM-CAGCCCGCACACCCGCATTAGG-TAMRA (probe); KDR, TCAAGATCCTCATCCACATTGG (forward), GGGCTTCGTGCAGGCA (reverse), FAM-CCA-TCTCAATGTGGTGAACCTGCTGG-TAMRA (probe); Flt, CCTCGCCAGAAGTCGTATGG (forward), CACCGAATAGC-GAGCAGATTT (reverse), FAM-TCCGTTGCGGGTACGC-CATGTT-TAMRA (probe); angiopoietin 1, AGATAACA-GAATCGCGTTCAAA (forward), TGAGACAAGAGGCTG-GTTCCTAT (reverse), FAM-CCACACGGCCACCATTGCTGG-TAMRA (probe); angiopoietin 2, TGGCTGGGCAACGAGTTT (forward), TGGATCTTCAGCACGTAGCG (reverse), FAM-TCTCCCAGCTGACCAGTGGGCA-TAMRA (probe); Tie-2, GGGTCGGTGGAATGACTT (forward), ACATTTGCTC-TCTTCAGTTGCAAC (reverse), FAM-CATCACCCGTGCTAT-TGGCGTTTCTGA-TAMRA (probe); eNOS, GTGCTGGC-ATACAGAACCCA (forward), CCATGTGGAACAGACCCCA (reverse), FAM-TGGGCTGGGCCCTCTGCACT-TAMRA (probe); and MCP-1, CAGATGCAGTTAATGCCCCAC (forward), AGC-CGACTCATTGGGATCAT (reverse), FAM-CACCTGCTGCTA-CTC-ATTCAGTGGCAA-TAMRA (probe). The specificity of each primer pair was evaluated using a 3% agarose gel; only a single product of appropriate size was produced for each PCR reaction. We verified excellent efficiency of the real-time PCR by showing a doubling of the products with each cycle over a 512-fold dilution range.

PCR (40 cycles) was performed in duplicate on 25 ng of cDNA from each sample using the Prism 7700 (Applied Biosystems) for real-time detection of PCR products. The fluorescence threshold (0.04) was set in the exponential phase of product formation. A reference sample of heart cDNA probed for glyceraldehyde-3-phosphate dehydrogenase was included on all plates (cycle time =  $24.6 \pm 0.11$ ) to verify consistency between plates.

#### Statistical Analysis

One-way ANOVA was used to assess the treatment effect of exercise training (SPSS version 9.0, SPSS; Chicago, IL). Tukey's *t*-test was used to detect significant differences between exercised animals and sedentary controls. *P* values  $< 0.05$  were considered significant.

Data are expressed as means  $\pm$  SE. No differences in mRNA expression over time were detected in sedentary Cont animals (Kruskal-Wallis nonparametric analysis). Thus the Cont animals were pooled into a single group for comparisons. While there were no differences in mRNA expression over time in the sedentary LIG animals (except for MCP-1

and angiopoietin 1), we have shown individual values in the figures.

## RESULTS

### Exercise Training

Average total run time for the rats increased rapidly for the first 8 days of the training program (Fig. 1). For the remainder of the training program, the rats maintained their level of performance of  $\sim 100$  min/day but did not further increase their running time. Thus it is likely that the strongest stimulus for angiogenesis occurred during the first 8 days of training.

### Muscle Capillarity

As illustrated in Fig. 2, angiogenesis was evident by a  $\sim 25\%$  increase ( $P < 0.001$ ) in muscle capillarity observed by day 12 of training. This increase in capillarity affected all fibers within the WG section, as the distribution of capillary contacts per fiber was uniformly shifted to higher values (Fig. 3). Thus fibers with relatively low numbers of capillary contacts increased in capillarity, as did the fibers with relatively high initial numbers of capillary contacts. No further increase in capillarity was observed with continued training after day 12. Muscle capillarity was not different between nonligated sedentary animals and ligated sedentary animals (Fig. 2, *inset*), and ligation alone did not alter the distribution of capillary contacts (Fig. 3, *inset*).

### Relative Expression of Angiogenic mRNAs in Control Muscle

Angiopoietin 1, angiopoietin 2, Tie-2, MCP-1, VEGF, KDR, Flt, and eNOS mRNA were readily detected in control muscles. Expression was found in all three of the muscle types studied: fast glycolytic (WG), fast oxidative (RG), and slow oxidative (SOL). The basal

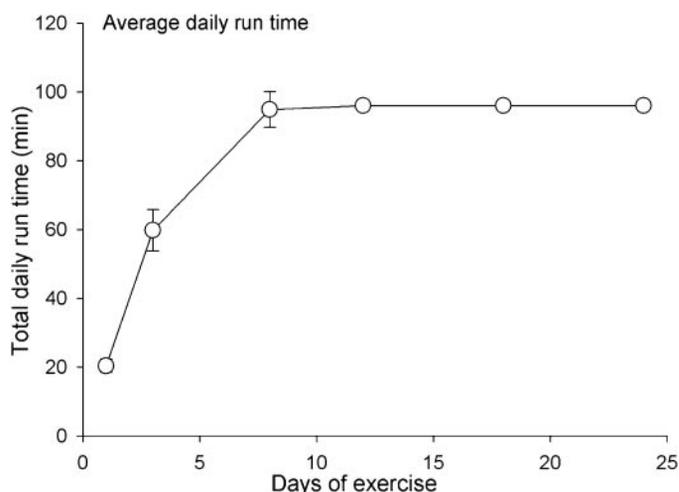


Fig. 1. Average total daily run times for trained rats. Exercise time and speed were recorded during the four daily runs for each rat. Data presented here are the total times run by the rats on the day they were euthanized (means  $\pm$  SE). Some error bars are covered by the symbol.  $n = 6$  (1, 18, and 24 days), 12 (3 and 8 days), and 5 (12 days).

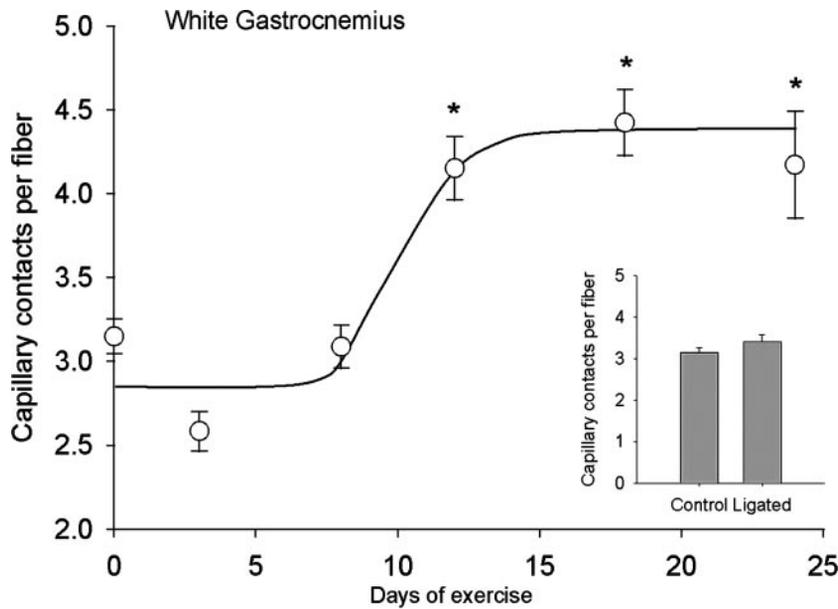


Fig. 2. Capillary contacts per fiber in the white gastrocnemius (WG) muscle section of exercise-trained ligated (LIG-EX) animals. *Inset*: average capillary contacts per fiber for the sedentary ligated (LIG) and control (Cont) animals. \*Significantly higher than other time points ( $P < 0.05$ ).

level of expression varied greatly among the growth factors and receptors studied (Fig. 4). In all three muscles, VEGF mRNA was by far the most abundant, and MCP-1 was the least abundant. In the WG and SOL muscles, the relative mRNA expression was VEGF  $\gggg$  Flt  $>$  angiopoietin 1  $>$  angiopoietin 2  $>$  Tie-2  $>$  eNOS  $>$  KDR  $>$  MCP-1. The same pattern was found in the RG muscle except that Tie-2  $>$  angiopoietin 2. In all three muscles, the angiopoietin 2-to-angiopoietin 1 ratio was  $<1$  (WG,  $0.87 \pm 0.08$ ; RG,  $0.40 \pm 0.03$ ; SOL,  $0.68 \pm 0.06$ ). Because angiopoietin 1 stabilizes vessels, whereas angiopoietin 2 destabilizes them, this ratio is consistent with a quiescent vasculature (26). Although the relative pattern of expression of the target mRNAs was similar for each fiber type, the magnitude of target expression varied with fiber type.

For all targets, expression was lower in the WG muscle than in the RG and SOL muscles (Fig. 4).

*Expression of Angiogenic mRNAs in Ligated-Only Muscle*

As may be expected in our model of acute-onset intermittent claudication, where flow capacity is well in excess of flow demands of resting muscle (51), ligation alone had no effect over time on the expression of the angiogenic mRNAs studied (Figs. 5 and 6), with the exception of a transient increase in MCP-1 mRNA (see below) and an apparent influence on angiopoietin 1 mRNA, which was uniformly reduced over time in the SOL muscle.

*Effect of Exercise Training on Angiogenic mRNA Expression*

*VEGF and VEGF receptors.* VEGF mRNA was up-regulated by exercise training (Fig. 5). In the WG

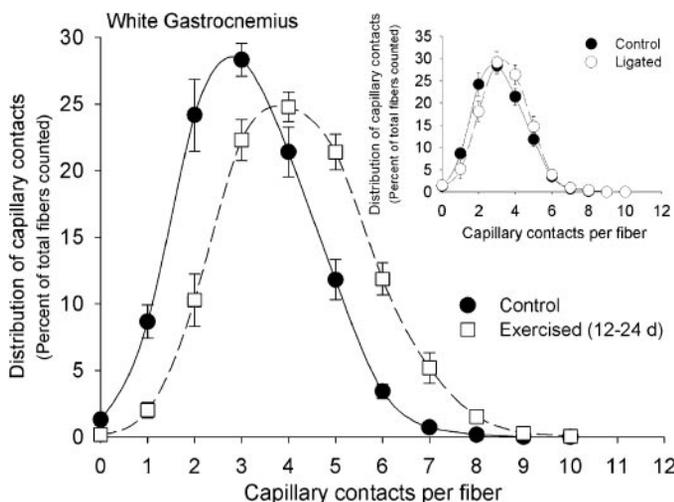


Fig. 3. Distribution of capillary contacts per fiber in the WG muscle section of LIG-EX and Cont animals. *Inset*: distribution of average capillary contacts per fiber for LIG and Cont animals.

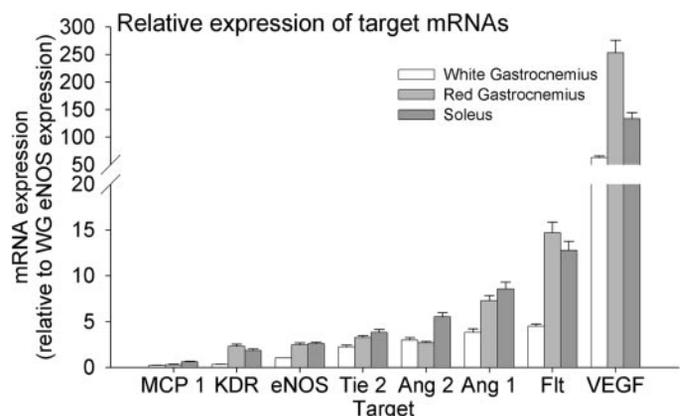


Fig. 4. Expression of target mRNAs in nonexercised Cont muscles. All targets are expressed relative to the abundance of endothelial nitric oxide synthase (eNOS) in WG muscles. Values are means  $\pm$  SE;  $n = 17$  for each. MCP-1, monocyte chemoattractant protein 1; Ang, angiopoietin.

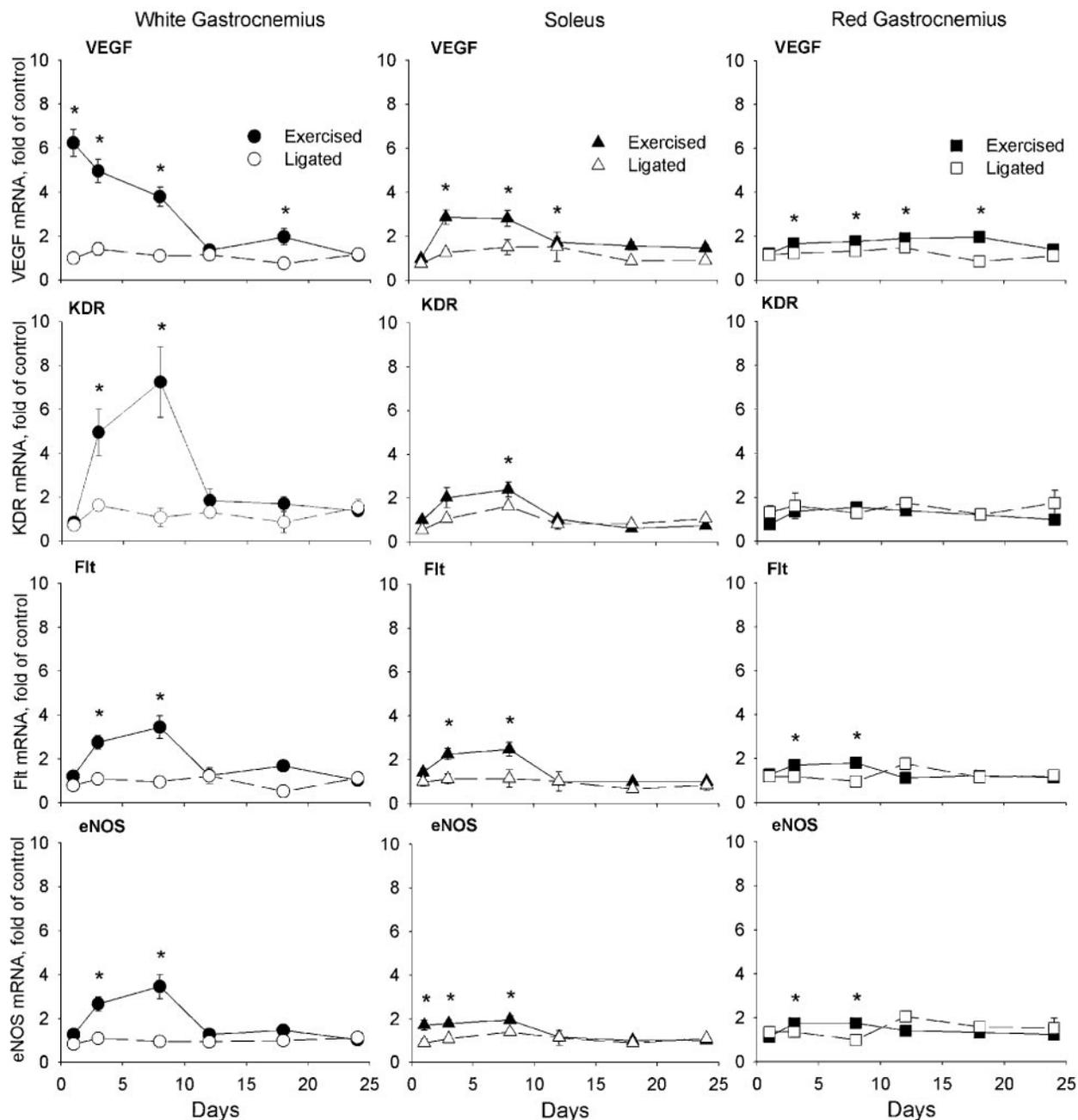


Fig. 5. Expression of VEGF, KDR, Flt, and eNOS mRNA in LIG-EX (solid symbols) and LIG (open symbols) rats (relative to the values in muscles of Cont animals). *Left*: WG muscle; *middle*: soleus (SOL) muscle; *right*, red gastrocnemius (RG) muscle. Values are means  $\pm$  SE. Some error bars are obscured by the symbols.  $n = 6$  (1, 12, 18, and 24 days) or 12 (3 and 8 days); LIG:  $n = 2-3$  per time point. \*Significantly higher than control ( $P < 0.05$ ).

muscle, VEGF mRNA was sharply increased after 1 day of training and declined toward control values thereafter. VEGF mRNA was also upregulated in the RG and SOL muscles. However, the pattern of expression in response to training was different from that observed in the WG muscle. In the RG muscle, the rise in VEGF mRNA did not occur until *day 3* of training. In addition, the elevation of VEGF mRNA in the RG muscle was both smaller and more sustained than that found in the WG muscle. The response of the SOL muscle was intermediate, with a moderate and tran-

sient increase in VEGF mRNA beginning at *day 3* (Fig. 5). The VEGF receptors KDR and Flt were also affected by exercise training. Again, the largest response was observed in the WG muscle, in which KDR mRNA expression peaked by *day 8* of training. Expression declined rapidly to control levels thereafter. Exercise did not affect KDR mRNA levels in the RG muscle. The response of the SOL muscle was intermediate between those of the WG and RG muscles, with a small elevation in KDR mRNA, which peaked at *day 8* (Fig. 5). The pattern of Flt expression was similar to that of KDR,

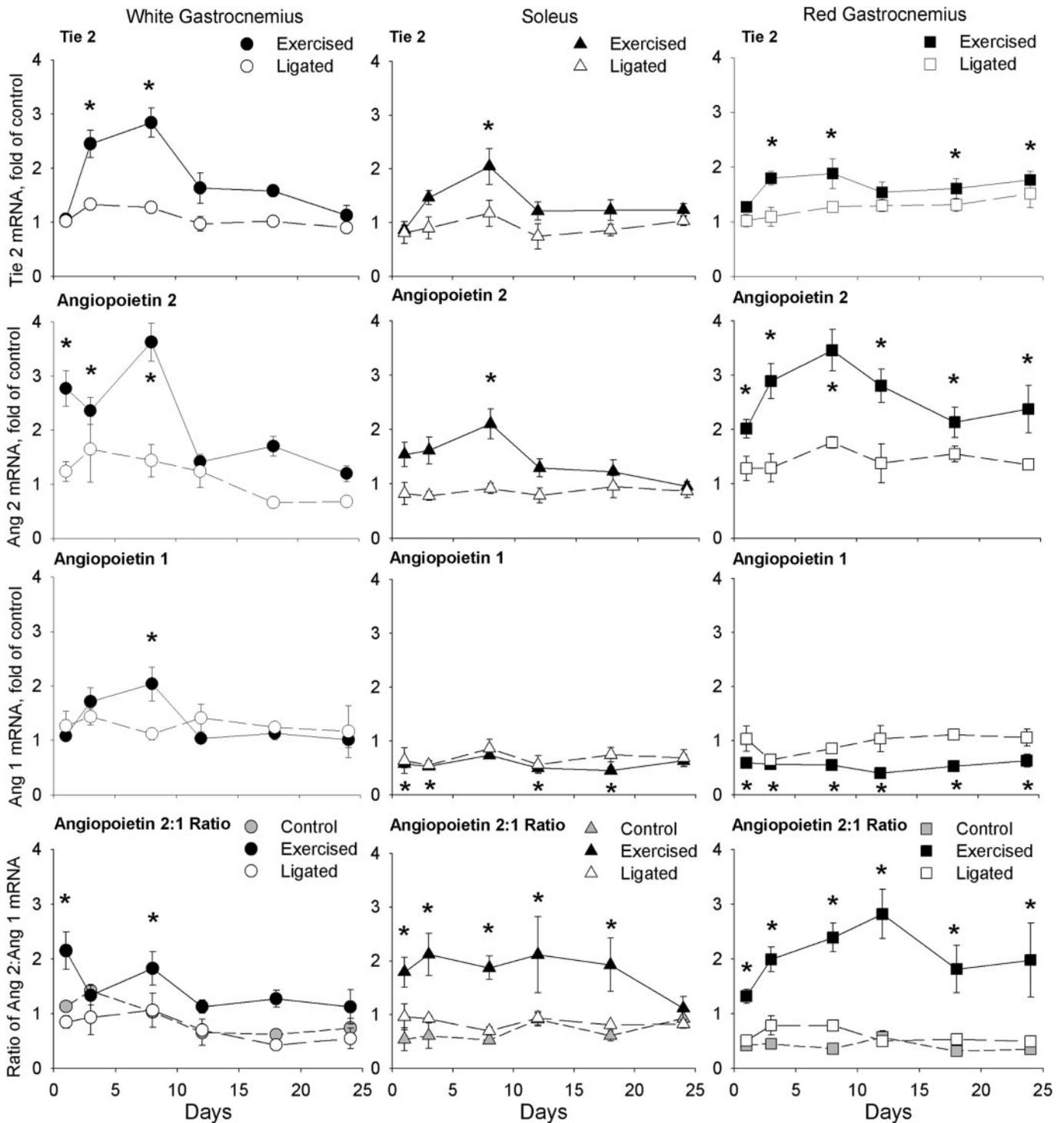


Fig. 6. *Top 3 rows*: expression of Tie-2, angiopoietin 2, and angiopoietin 1 mRNA in LIG-EX (solid symbols) and LIG (open symbols) rats (relative to the values in Cont animals). *Bottom*: absolute ratio of angiopoietin 2 to angiopoietin 1, with Cont values shown in gray (because values are not normalized to Cont values). *Left*, WG muscle; *middle*, SOL muscle; *right*, RG muscle. Values are means  $\pm$  SE. Some error bars are obscured by the symbols.  $n = 6$  (1, 12, 18, and 24 days) or 12 (3 and 8 days); LIG:  $n = 2-3$  per time point. \*Significantly different from control ( $P < 0.05$ ).

with the response being WG > SOL > RG. Flt mRNA expression followed a similar time course to KDR mRNA expression, peaking by days 3-8 of training (Fig. 5).

*eNOS expression.* Training increased eNOS mRNA in all three muscles. The response to training was greatest in the WG muscle and least in the RG muscle. The peak of eNOS mRNA expression occurred by 8

days of training. Levels returned to control thereafter (Fig. 5).

**Angiopoietins and Tie-2.** Expression of mRNA for angiopoietins 1 and 2 and their receptor Tie-2 was affected by exercise training. As illustrated in Fig. 6, training increased the angiopoietin 2-to-angiopoietin 1 ratio in all three fiber sections, primarily due to an increase in angiopoietin 2, although the decrease in angiopoietin 1 in the RG and SOL muscles also contributed. Interestingly, the fiber-type pattern of the angiopoietin response was reversed relative to the pattern found for VEGF and VEGF receptors. Training had the largest effect on the ratio in the RG muscle, an intermediate effect in the SOL muscle, and the smallest effect in the WG muscle (Fig. 6). Tie-2 mRNA was also upregulated by training. As for VEGF, KDR, and Flt, the largest increase in Tie-2 mRNA was found in the WG muscle. In all three muscles, Tie-2 mRNA expression peaked by *day 8* of training (Fig. 6). It remained elevated thereafter only in the RG section.

**MCP-1 expression.** The most striking change in mRNA expression in response to training was found in MCP-1 mRNA, in which a sharp elevation was evident after 1 day of training in all three muscles (Fig. 7). The largest effect of training occurred in the RG muscle and the smallest effect was found in the WG muscle, as for the angiopoietin 2-to-angiopoietin 1 ratio, and in contrast to the other factors studied. Expression declined rapidly after *day 1*. MCP-1 mRNA was also elevated by ligation alone in the RG and SOL muscles (but not in the WG muscle), with the largest effect seen 1 day after ligation (RG,  $8.78 \pm 1.73$ -fold increase; SOL,  $12.55 \pm 4.74$ -fold increase). However, these responses were much less pronounced in the LIG rats than in the LIG-EX rats. For example, on *day 1*, MCP-1 mRNA levels were  $\sim 6.5$ -fold higher in the RG muscle and  $\sim 3$ -fold higher in the SOL muscle of LIG-EX rats relative to LIG rats.

## DISCUSSION

This study was designed to investigate the effects of ischemic exercise training on the expression of a number of important growth factors that have been implicated in vascular remodeling. We provide the first evidence that activation of the angiopoietin and VEGF pathways occurs in skeletal muscle in response to exercise training, in a manner that appears coordinated for angiogenesis. As discussed below, changes in gene expression are most dramatic during the initial phase of training, are tempered as the training progresses, and finally reach an apparent steady state. Because angiogenesis is histologically evident by *day 12*, the angiogenic process is likely to be ongoing during the initial period of training, when changes in gene expression are most marked. This is most evident in the low oxidative, low vascular WG section, in which increased capillarity is preceded by both enhanced VEGF/VEGF receptor mRNA abundance and changes in the angiopoietin 2-to-angiopoietin 1 mRNA ratio.

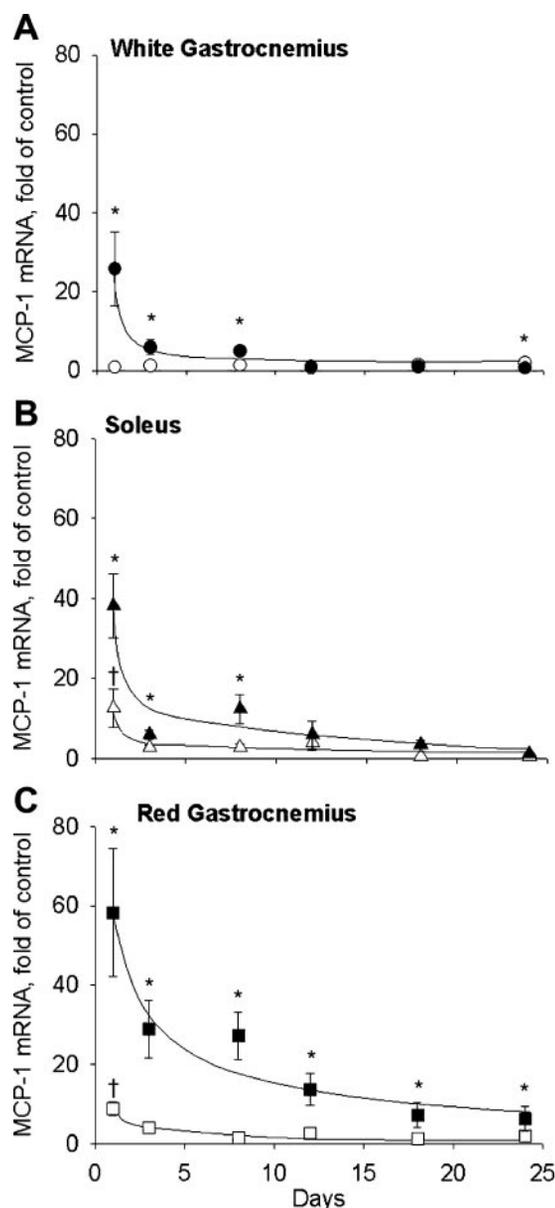


Fig. 7. Expression of MCP-1 mRNA in LIG-EX (solid symbols) and LIG (open symbols) rats relative to the response in muscles of Cont animals. A: WG muscle; B: SOL muscle; C: RG muscle. Values are means  $\pm$  SE. Lines were drawn by hand to aid the eye. Some error bars are obscured by the symbols.  $n = 6$  (1, 12, 18, and 24 days) or 12 (3 and 8 days); LIG:  $n = 2-3$  per time point. \*LIG-EX values significantly different from control ( $P < 0.05$ ); †LIG values significantly different from control ( $P < 0.05$ ).

These shifts in gene expression should favor angiogenesis.

### Exercise Training Activates Multiple Growth Factor Pathways

**VEGF and VEGF receptors.** In the WG muscle, there was a large initial increase in VEGF mRNA, followed by a progressive decline to control values over the remainder of the training program. This pattern confirms previous observations after exercise in non-trained rats (4) and during training in humans (32).

The increase in VEGF mRNA observed with exercise (13) and/or muscle contractions (1) is associated with an increase in VEGF protein. Thus increased VEGF protein levels should provide an important stimulus for angiogenesis, especially during the early phase of the training program. Interestingly, there was no detectable increase in muscle capillarity during the early time period of training, when the VEGF mRNA changes were most apparent. Rather, a robust increase in capillarity was observed beginning at *day 12* of training (cf. Fig. 2). Because angiogenesis has been observed as early as 5 days after the onset of chronic muscle stimulation, it is likely that the angiogenic process was already ongoing at earlier time points, during the dominant phase of the VEGF response. However, a sustained exercise stimulus, occurring over a number of days, may be necessary in order for the development of angiogenesis to become fully manifest.

It is presently unclear whether the tempered response of VEGF mRNA to continued exercise training is due to reduced transcription and/or an altered half-life of the message, because both of these mechanisms are known to regulate VEGF mRNA levels (39). Furthermore, it is unclear what metabolic and/or hemodynamic stimuli prompt VEGF mRNA upregulation in response to exercise. Hypoxia is a major regulator of VEGF mRNA expression (38) and cannot be dismissed as an important contributor to the responses observed in this study. However, our experiments were not designed to "factor out" the potential influence of hypoxia (ischemia). We did not include a nonligated trained group, because definitive conclusions about the exercise response in these animals would have been preempted by complexities associated with motor unit recruitment during exercise. For example, to match the exercise stimulus between groups, nonligated animals would have had to run at the same low speed as the ligated animals. However, in nonligated animals, this exercise intensity would not meaningfully recruit fast-twitch white motor units (7). Thus any differences in gene expression between the nonligated exercised and ligated exercised WG muscle sections would reflect altered recruitment rather than the metabolic and/or signaling consequences of myocyte hypoxia.

Although hypoxia is a known regulator of VEGF mRNA, Gustafsson et al. (14) have questioned its role in the exercise response. Indeed, Breen et al. (4) reported a significant elevation in VEGF mRNA in normal animals during exercise conditions that are not expected to produce frank hypoxia, suggesting that other signals associated with muscle contraction are sufficient to elevate VEGF mRNA. These signals could be produced in response to flow-dependent stimuli and/or mechanical changes induced by load bearing within the muscle during exercise. For example, increased stretch has been shown to increase VEGF mRNA expression in skeletal muscle (33), cardiac myocytes (53), and the intact heart (52). Likewise, increased shear stress during exercise may play an important role in modulating angiogenic factor gene expression. Shear stress is known to increase NO levels

(29), and it has recently been shown that NO can activate hypoxia-inducible factor 1, a major regulator of VEGF expression, in the absence of hypoxia (21). Thus while hypoxia, shear stress, and mechanical stimuli are all potential stimulators of VEGF gene expression in response to exercise, it is presently unknown which of these stimuli actually account for the enhanced abundance of this important angiogenic growth factor in our model.

Training also increased the abundance of VEGF receptor mRNAs (KDR and Flt). However, the timing of KDR and Flt mRNA upregulation was delayed relative to the increase in VEGF mRNA. KDR and Flt mRNA levels were not elevated initially but began rising by *day 3* of training and peaked by *day 8*. Although we did not evaluate VEGF receptor protein levels, other studies have shown a correspondence between elevated mRNA levels and increased protein expression for both KDR (36) and Flt (41). The delayed response of KDR and Flt mRNA to training suggests that tissue ischemia is not the primary regulator of these mRNAs, because ischemia should be evident from the first day. Perhaps the delay in upregulation of these receptor mRNAs, relative to the increase in VEGF, is a control mechanism that moderates the response to VEGF, preventing brief periods of increased VEGF expression from resulting in inappropriate angiogenesis.

*Angiopoietins and Tie-2.* In addition to its effect on the VEGF pathway, exercise training also altered the expression of mRNAs in the angiopoietin signaling pathway. Recent evidence suggests that the angiopoietins play crucial roles in modulating VEGF-induced angiogenesis. Both angiopoietins bind to the Tie-2 receptor and thus compete with each other. Angiopoietin 1 promotes vessel stability (40), whereas angiopoietin 2 has the opposite effect (26). Therefore, the ratio of angiopoietin 2-to-angiopoietin 1 is thought to determine whether the net effect of the angiopoietins is to stabilize or destabilize the vasculature, with an increase in the angiopoietin 2-to-angiopoietin 1 ratio (destabilization) being proangiogenic. For example, angiopoietin 2 is upregulated at the leading edge of new vessel growth (26). Furthermore, studies in transgenic mouse myocardium show that angiopoietin 2 has a synergistic effect with VEGF on capillary growth, whereas angiopoietin 1 antagonizes VEGF action (42). Thus a balance between these three factors appears to be necessary for normal vessel growth. Finally, studies in tumors suggest that angiopoietin 2 expression in the presence of VEGF leads to vessel growth, but angiopoietin 2 expressed in the absence of VEGF causes vessel regression (17). In our model, the angiopoietin 2-to-angiopoietin 1 mRNA ratio was elevated after a single day of exercise, suggesting that destabilization of the existing muscle vasculature is an early event in exercise-induced angiogenesis. Interestingly, although we found that angiopoietin receptor (Tie-2) mRNA was also increased, the upregulation of receptor mRNA was delayed relative to the changes in ligand mRNA (as was found for the VEGF system). Importantly, the initial increase in the angiopoietin 2-to-angiopoietin 1

mRNA ratio coincided with the upregulation of VEGF mRNA. This conforms to the pattern apparently needed to foster expansion of the capillary network. Thus we show for the first time that an apparent coordinated activation of VEGF and the angiotensin system occurs during physiological angiogenesis in skeletal muscle.

*eNOS.* Training also elevated eNOS mRNA in the WG muscle, with a time course similar to that of the three receptors studied. Numerous studies have suggested that NO is a component of the VEGF signaling pathway (19, 31, 54) and thus is potentially important in angiogenesis (6, 30, 37). Exercise training is known to increase NO pathway activity (12, 22, 24, 43), and eNOS knockout mice show a requirement for NO in skeletal muscle angiogenesis (28), suggesting that angiogenesis in response to exercise might involve NO. In keeping with this idea, NOS inhibition tempers the upregulation of both VEGF (10) and Flt (11) by exercise and eliminates the increase in muscle capillarity typically observed with chronic low-frequency stimulation (20). However, these studies did not directly assess the role of NO in exercise-stimulated angiogenesis. We have recently shown that inhibition of NO synthesis with  $N^G$ -nitro-L-arginine methyl ester (L-NAME) does not block exercise-induced angiogenesis in the rat ischemic hindlimb (although it does inhibit arteriogenesis, the remodeling of collateral conduit arteries) (25). This finding suggests that NO is not involved in exercise-stimulated angiogenesis or, alternatively, that compensatory mechanisms exist that can stimulate angiogenesis in the absence of intact NO synthesis capability.

Our current data showing that eNOS mRNA is upregulated in response to exercise with a time course similar to other components of the VEGF signaling pathway (KDR and Flt) support the idea that NO is involved in training-induced angiogenesis. Thus our previous finding that angiogenic capability is retained in skeletal muscle of rats subjected to NO synthesis inhibition may reflect either activation of alternate, NO-independent angiogenic mechanisms in skeletal muscle or enhanced eNOS gene expression during training resulting in an enhanced capacity for NO production, even in the face of L-NAME. Finally, it is possible that the upregulation of eNOS in response to training serves a function unrelated to angiogenesis (e.g., enhanced endothelial-mediated responsiveness). Thus further studies are necessary before definitive conclusions can be drawn about the role of NO in the angiogenic response to training.

*MCP-1.* Training increased MCP-1 mRNA in the WG muscle, with a time course similar to that of VEGF mRNA. The signaling mechanism mediating this increase is unknown. However, the transcription factors nuclear factor (NF)- $\kappa$ B and activator protein (AP)-1 are both recognized regulators of MCP-1 transcription (23), and increased binding of NF- $\kappa$ B and AP-1 has been implicated in the upregulation of superoxide dismutase mRNA by exercise (18). Thus exercise could potentially induce MCP-1 mRNA via NF- $\kappa$ B and/or AP-1. VEGF

could also contribute to the enhanced MCP-1 mRNA levels, because it stimulates MCP-1 production in endothelial cells (27).

Although MCP-1 has been implicated in arteriogenesis after occlusion of a major supply vessel (16), its exact function in angiogenesis is unclear. MCP-1 has recently been shown to have a direct angiogenic effect on endothelial cells (34). MCP-1 can also indirectly stimulate angiogenesis by recruiting monocytes to the tissue, where they release angiogenic growth factors such as tumor necrosis factor- $\alpha$  and basic fibroblast growth factor (2). Thus the finding that MCP-1 expression is strikingly elevated after 1 day of exercise may indicate that monocyte-derived growth factors are involved in exercise-induced angiogenesis. If important, this response appears to be tempered with continued exercise over the training period.

The timing of MCP-1 expression during vascular remodeling appears to be key (16). Our data showing that MCP-1 mRNA increased immediately after initiation of the exercise program and then declined but remained above control levels for several days suggest that MCP-1 may have different functions at different times in the angiogenic process. For instance, an early effect of MCP-1 could be to recruit monocytes to the area, resulting in increased levels of a variety of growth factors. A later angiogenic effect of MCP-1 may be to stimulate endothelial cell migration, because MCP-1 can induce endothelial cell chemotaxis (34).

#### *Fiber-Type Differences in Angiogenic Response to Exercise*

Our hypothesis that the upregulation of angiogenic factors would be greatest in the low-capillarity WG muscle section was only partially supported. The increases in VEGF, VEGF receptors, Tie-2, and eNOS mRNA expression with exercise training in the different fiber types tended to scale inversely with muscle vascularity. In contrast, the fiber-type pattern of response for the angiotensin 2-to-angiotensin 1 ratio during training was opposite to our expectations. Training affected the angiotensin 2-to-angiotensin 1 ratio more in the high-oxidative than in the low-oxidative fiber sections. This finding implies that the responsiveness to vascular remodeling may be greatest in the highly vascularized sections. However, as discussed above, the presence or absence of VEGF determines whether angiogenesis will occur when angiotensin 2 is dominant over angiotensin 1. In this regard, it is worth noting that the VEGF response in the RG muscle was muted relative to the response in the WG muscle, and thus angiogenesis would not be expected to occur in this environment. Interestingly, we previously observed that the RG muscle section did not exhibit any increase in capillarity in response to a similar training program as used in this study (50). Thus the significance of the changes in the angiotensin 2-to-angiotensin 1 ratio in the RG muscle is presently unclear.

The MCP-1 results also contradicted our hypothesis. Training increased MCP-1 mRNA more in the high-

oxidative than in the low-oxidative fiber sections, possibly in response to more sustained recruitment of the motor units during the exercise bout. Similarly, MCP-1 mRNA was elevated in the RG and SOL muscle, but not in the WG muscle, of cage-restricted sedentary LIG rats (albeit to a much lesser extent than in LIG-EX rats). This pattern of response suggests that cage activity, during which RG and SOL (but not WG) motor units would likely be utilized (5), is a sufficient stimulus to upregulate MCP-1, at least in the brief period after occlusion of the femoral arteries.

In conclusion, in the present study, we provide the first evidence to our knowledge that expression of the angiopoietins and Tie-2 are altered in skeletal muscle by exercise training. The increased angiopoietin 2-to-angiopoietin 1 ratio should have a permissive effect on angiogenesis by allowing the early increase in VEGF to initiate new capillary growth, which is apparent by day 12. Although the response of VEGF and its receptors was tempered later in the training program, muscle capillarity remained elevated. These results suggest that exercise-induced angiogenesis involves coordinated activation of both the angiopoietin and VEGF signaling pathways. Changes in eNOS and MCP-1 mRNA expression also implicate these factors in the angiogenic response to training. These findings highlight the complex nature of physiological angiogenesis.

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## REFERENCES

- Annex BH, Torgan CE, Lin P, Taylor DA, Thompson MA, Peters KG, and Kraus WE. Induction and maintenance of increased VEGF protein by chronic motor nerve stimulation in skeletal muscle. *Am J Physiol Heart Circ Physiol* 274: H860–H867, 1998.
- Arras M, Ito WD, Scholz D, Winkler B, Schaper J, and Schaper W. Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. *J Clin Invest* 101: 40–50, 1998.
- Asahara T, Chen D, Takahashi T, Fujikawa K, Kearney M, Magner M, Yancopoulos GD, and Isner JM. Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. *Circ Res* 83: 233–240, 1998.
- Breen EC, Johnson EC, Wagner H, Tseng HM, Sung LA, and Wagner PD. Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. *J Appl Physiol* 81: 355–361, 1996.
- Burke RE and Edgerton VR. Motor unit properties and selective involvement in movement. *Exerc Sport Sci Rev* 3: 31–83, 1975.
- Dimmeler S, Dernbach E, and Zeiher AM. Phosphorylation of the endothelial nitric oxide synthase at ser-1177 is required for VEGF-induced endothelial cell migration. *FEBS Lett* 477: 258–262, 2000.
- Dudley GA, Abraham WM, and Terjung RL. Influence of exercise intensity and duration on biochemical adaptations in skeletal muscle. *J Appl Physiol* 53: 844–850, 1982.
- Ernst E. Peripheral vascular disease. Benefits of exercise. *Sports Med* 12: 149–151, 1991.
- Gavin TP, Spector DA, Wagner H, Breen EC, and Wagner PD. Effect of captopril on skeletal muscle angiogenic growth factor responses to exercise. *J Appl Physiol* 88: 1690–1697, 2000.
- Gavin TP, Spector DA, Wagner H, Breen EC, and Wagner PD. Nitric oxide synthase inhibition attenuates the skeletal muscle VEGF mRNA response to exercise. *J Appl Physiol* 88: 1192–1198, 2000.
- Gavin TP and Wagner PD. Attenuation of the exercise-induced increase in skeletal muscle Flt-1 mRNA by nitric oxide synthase inhibition. *Acta Physiol Scand* 175: 201–209, 2002.
- Griffin KL, Laughlin MH, and Parker JL. Exercise training improves endothelium-mediated vasorelaxation after chronic coronary occlusion. *J Appl Physiol* 87: 1948–1956, 1999.
- Gustafsson T, Knutsson A, Puntchart A, Kaijser L, Nordqvist AC, Sundberg CJ, and Jansson E. 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, 2002.
- Gustafsson T, Puntchart A, Kaijser L, Jansson E, and Sundberg CJ. Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. *Am J Physiol Heart Circ Physiol* 276: H679–H685, 1999.
- Gute D, Fraga C, Laughlin MH, and Amann JF. Regional changes in capillary supply in skeletal muscle of high-intensity endurance-trained rats. *J Appl Physiol* 81: 619–626, 1996.
- Hoefler IE, van Royen N, Buschmann IR, Piek JJ, and Schaper W. Time course of arteriogenesis following femoral artery occlusion in the rabbit. *Cardiovasc Res* 49: 609–617, 2001.
- Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, and Wiegand SJ. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science* 284: 1994–1998, 1999.
- Hollander J, Fiebiger R, Gore M, Ookawara T, Ohno H, and Ji LL. Superoxide dismutase gene expression is activated by a single bout of exercise in rat skeletal muscle. *Pflügers Arch* 442: 426–434, 2001.
- Hood JD, Meininger CJ, Ziche M, and Granger HJ. VEGF upregulates eNOS message, protein, and NO production in human endothelial cells. *Am J Physiol Heart Circ Physiol* 274: H1054–H1058, 1998.
- Hudlicka O, Brown MD, and Silgram H. Inhibition of capillary growth in chronically stimulated rat muscles by  $N^G$ -nitro-L-arginine, nitric oxide synthase inhibitor. *Microvasc Res* 59: 45–51, 2000.
- Kimura H, Weisz A, Kurashima Y, Hashimoto K, Ogura T, D'Acquisto F, Addeo R, Makuuchi M, and Esumi H. Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. *Blood* 95: 189–197, 2000.
- Koller A, Huang A, Sun D, and Kaley G. Exercise training augments flow-dependent dilation in rat skeletal muscle arterioles. Role of endothelial nitric oxide and prostaglandins. *Circ Res* 76: 544–550, 1995.
- Lakshminarayanan V, Lewallen M, Frangogiannis NG, Evans AJ, Wedin KE, Michael LH, and Entman ML. Reactive oxygen intermediates induce monocyte chemotactic protein-1 in vascular endothelium after brief ischemia. *Am J Pathol* 159: 1301–1311, 2001.
- Laughlin MH, Pollock JS, Amann JF, Hollis MH, Woodman CR, and Price EM. Training induces nonuniform increases in eNOS content along the coronary arterial tree. *J Appl Physiol* 90: 501–510, 2001.
- Lloyd PG, Yang HT, and Terjung RL. Arteriogenesis and angiogenesis in rat ischemic hindlimb: role of nitric oxide. *Am J Physiol Heart Circ Physiol* 281: H2528–H2538, 2001.
- Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, and Yancopoulos GD. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277: 55–60, 1997.
- Marumo T, Schini-Kerth VB, and Busse R. Vascular endothelial growth factor activates nuclear factor-kappaB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. *Diabetes* 48: 1131–1137, 1999.
- Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC,

- Huang PL, and Isner JM.** Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 101: 2567–2578, 1998.
29. **Noris M, Morigi M, Donadelli R, Aiello S, Foppolo M, Todeschini M, Orisio S, Remuzzi G, and Remuzzi A.** Nitric oxide synthesis by cultured endothelial cells is modulated by flow conditions. *Circ Res* 76: 536–543, 1995.
  30. **Papapetropoulos A, Garcia-Cardena G, Madri JA, and Sessa WC.** Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* 100: 3131–3139, 1997.
  31. **Parenti A, Morbidelli L, Cui XL, Douglas JG, Hood JD, Granger HJ, Ledda F, and Ziche M.** Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium. *J Biol Chem* 273: 4220–4226, 1998.
  32. **Richardson RS, Wagner H, Mudaliar SR, Saucedo E, Henry R, and Wagner PD.** Exercise adaptation attenuates VEGF gene expression in human skeletal muscle. *Am J Physiol Heart Circ Physiol* 279: H772–H778, 2000.
  33. **Rivlis I, Milkiewicz M, Boyd P, Goldstein J, Brown MD, Egginton S, Hansen FM, Hudlicka O, and Haas TL.** Differential involvement of MMP-2 and VEGF during muscle stretch-versus shear stress-induced angiogenesis. *Am J Physiol Heart Circ Physiol* 283: H1430–H1438, 2002.
  34. **Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, and Murphy WJ.** Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* 96: 34–40, 2000.
  35. **Seligman AM, Heymann H, and Barnett RJ.** The histochemical demonstration of alkaline phosphatase activity with indoxyl phosphate. *J Histochem Cytochem* 2: 441–442, 1954.
  36. **Shen BQ, Lee DY, Gerber HP, Keyt BA, Ferrara N, and Zioncheck TF.** Homologous up-regulation of KDR/Flk-1 receptor expression by vascular endothelial growth factor in vitro. *J Biol Chem* 273: 29979–29985, 1998.
  37. **Shizukuda Y, Tang S, Yokota R, and Ware JA.** Vascular endothelial growth factor-induced endothelial cell migration and proliferation depend on a nitric oxide-mediated decrease in protein kinase C $\delta$  activity. *Circ Res* 85: 247–256, 1999.
  38. **Shweiki D, Itin A, Soffer D, and Keshet E.** Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359: 843–845, 1992.
  39. **Stein I, Neeman M, Shweiki D, Itin A, and Keshet E.** Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregulation with other ischemia-induced genes. *Mol Cell Biol* 15: 5363–5368, 1995.
  40. **Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, and Yancopoulos GD.** Requirement of angiopoietin-1, a ligand for the Tie2 receptor, during embryonic angiogenesis. *Cell* 87: 1171–1180, 1996.
  41. **Vidal F, Aragones J, Alfranca A, and de Landazuri MO.** Up-regulation of vascular endothelial growth factor receptor Flt-1 after endothelial denudation: role of transcription factor Egr-1. *Blood* 95: 3387–3395, 2000.
  42. **Visconti RP, Richardson CD, and Sato TN.** Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). *Proc Natl Acad Sci USA* 99: 8219–8224, 2002.
  43. **Woodman CR, Muller JM, Laughlin MH, and Price EM.** Induction of nitric oxide synthase mRNA in coronary resistance arteries isolated from exercise-trained pigs. *Am J Physiol Heart Circ Physiol* 273: H2575–H2579, 1997.
  44. **Yang HT, Deschenes MR, Ogilvie RW, and Terjung RL.** Basic fibroblast growth factor increases collateral blood flow in rats with femoral arterial ligation. *Circ Res* 79: 62–69, 1996.
  45. **Yang HT, Dinn RF, and Terjung RL.** Training increases muscle blood flow in rats with peripheral arterial insufficiency. *J Appl Physiol* 69: 1353–1359, 1990.
  46. **Yang HT, Feng Y, Allen LA, Protter A, and Terjung RL.** Efficacy and specificity of bFGF increased collateral flow in experimental peripheral arterial insufficiency. *Am J Physiol Heart Circ Physiol* 278: H1966–H1973, 2000.
  47. **Yang HT, Ogilvie RW, and Terjung RL.** Low-intensity training produces muscle adaptations in rats with femoral artery stenosis. *J Appl Physiol* 71: 1822–1829, 1991.
  48. **Yang HT, Ogilvie RW, and Terjung RL.** Peripheral adaptations in trained aged rats with femoral artery stenosis. *Circ Res* 74: 235–243, 1994.
  49. **Yang HT, Ogilvie RW, and Terjung RL.** Training increases collateral-dependent muscle blood flow in aged rats. *Am J Physiol Heart Circ Physiol* 268: H1174–H1180, 1995.
  50. **Yang HT, Ogilvie RW, and Terjung RL.** Heparin increases exercise-induced collateral blood flow in rats with femoral artery ligation. *Circ Res* 76: 448–456, 1995.
  51. **Yang HT, Ogilvie RW, and Terjung RL.** Exercise training enhances basic fibroblast growth factor-induced collateral blood flow. *Am J Physiol Heart Circ Physiol* 274: H2053–H2061, 1998.
  52. **Zheng W, Brown MD, Brock TA, Bjercke RJ, and Tomanek RJ.** Bradycardia-induced coronary angiogenesis is dependent on vascular endothelial growth factor. *Circ Res* 85: 192–198, 1999.
  53. **Zheng W, Seftor EA, Meininger CJ, Hendrix MJ, and Tomanek RJ.** Mechanisms of coronary angiogenesis in response to stretch: role of VEGF and TGF- $\beta$ . *Am J Physiol Heart Circ Physiol* 280: H909–H917, 2001.
  54. **Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S, Granger HJ, and Bicknell R.** Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest* 99: 2625–2634, 1997.