

Research article

Regional differences in expression of VEGF mRNA in rat gastrocnemius following 1 hr exercise or electrical stimulation

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Abstract

Background: Vascular endothelial growth factor (VEGF) mRNA levels increase in rat skeletal muscle after a single bout of acute exercise. We assessed regional differences in VEGF₁₆₅ mRNA levels in rat gastrocnemius muscle using *in situ* hybridization after inducing upregulation of VEGF by treadmill running (1 hr) or electrical stimulation (1 hr). Muscle functional regions were defined as oxidative (primarily oxidative fibers, I and IIa), or glycolytic (entirely IIb or IIc/x fibers). Functional regions were visualized on muscle cross sections that were matched in series to slides processed through *in situ* hybridization with a VEGF₁₆₅ probe. A greater upregulation in oxidative regions was hypothesized.

Results: Total muscle VEGF mRNA (via Northern blot) was upregulated 3.5-fold with both exercise and with electrical stimulation ($P = 0.015$). Quantitative densitometry of the VEGF mRNA signal via *in situ* hybridization reveals significant regional differences ($P \leq 0.01$) and protocol differences (treadmill, electrical stimulation, and control, $P \leq 0.05$). Mean VEGF mRNA signal was higher in the oxidative region in both treadmill run (~7%, $N = 4$ muscles, $P \leq 0.05$) and electrically stimulated muscles (~60%, $N = 4$, $P \leq 0.05$). These regional differences were not significantly different from control muscle (non-exercised, non-stimulated, $N = 2$ muscles), although nearly so for electrically stimulated muscle ($P = 0.056$).

Conclusions: Moderately higher VEGF mRNA signal in oxidative muscle regions is consistent with regional differences in capillary density. However, it is not possible to determine if the VEGF mRNA signal difference is important in either the maintenance of regional capillarity differences or exercise induced angiogenesis.

Background

The adaptations of skeletal muscle to endurance-type training are well characterized. They include increases in mitochondrial volume density and increases in the activi-

ty of enzymes involved in oxidative phosphorylation to produce ATP [1]. The increased metabolic capacity of trained muscle is accompanied by an angiogenic response which increases capillary density and/or capillary to fiber

ratio [2,3], preserving the functional match between oxygen delivery and metabolic demand within the muscle. The angiogenic response in skeletal muscle is thought to be mediated by a number of angiogenic factors including, most importantly, vascular endothelial growth factor (VEGF). VEGF is a 45 kDa heparin-binding homodimeric glycoprotein with a predominant specificity to vascular endothelial cells [4–7]. Recent investigations demonstrate that VEGF increases vascular permeability [4], endothelial cell proliferation *in vitro* [8], and angiogenesis *in vivo* [9]. We have previously demonstrated that VEGF mRNA is up-regulated in rat gastrocnemius muscle following 1 hour of acute submaximal treadmill exercise [10], in dog gastrocnemius muscle following 1 hour of electrical stimulation [11], and in human vastus lateralis following 30 min of one-legged knee extension exercise at 50% maximal capacity [12]. Other studies show similar upregulation of VEGF mRNA in chronically electrically stimulated rat skeletal muscle [13,14], and in human vastus lateralis following 45 min of one-legged knee extension exercise [15].

VEGF mRNA transcripts are produced within skeletal muscle fibers and may localize in the sub-sarcolemmal region [10], but it is unknown whether differences in VEGF mRNA expression exist between functionally diverse muscle regions with markedly different fiber type compositions. Because of capillary density and metabolic differences between oxidative and glycolytic muscle regions, we hypothesize a greater up-regulation of VEGF mRNA in oxidative regions of a mixed skeletal muscle. The rat gastrocnemius is an ideal muscle to investigate these questions as it is comprised of approximately 28% fast oxidative glycolytic (FOG), 65% fast glycolytic (FG), and 7% slow (S) muscle fibers [16], according to a metabolic enzyme-based fiber type classification scheme]. Importantly, this muscle also shows significant regionalization i.e., deep versus superficial regions of both medial and lateral heads of the rat gastrocnemius can be characterized as oxidative (predominantly S and FOG fibers) or glycolytic (predominantly FG fibers), respectively [16].

In the present study we used a 1 hr. treadmill exercise protocol or a 1 hr. electrical stimulation protocol to upregulate total VEGF mRNA production in rat gastrocnemius muscle. Both protocols have previously been demonstrated to upregulate VEGF mRNA levels by 3–4 fold [10,13,14]. *In situ* hybridization with a VEGF₁₆₅ probe was then applied to muscle samples to detect the fiber specific localization of VEGF mRNA transcripts. In order to observe the pattern of regional VEGF production, serial cross sections were used to identify fiber types as I, IIa, IIb, and IIc/x by histochemistry and immunohistochemistry. Under this classification scheme, fibers are characterized as follows: type I fibers are slow-twitch with a high oxida-

tive capacity; type IIa fibers are fast-twitch with a high oxidative and glycolytic capacity; type IIb fibers are fast-twitch with a low oxidative and a high glycolytic capacity; and type IIc/x fibers (common in small mammals) are fast-twitch with a metabolic profile intermediate to that of IIa and IIb fibers [17].

Results

Determination of fiber types and VEGF mRNA signal on serial slide sections

Figure 1 shows serial sections of rat gastrocnemius after 1 hour of treadmill running. The VEGF mRNA signal is apparent as a dark stain (panel A), and may be compared to the sense control (panel B) which shows no non-specific binding in the same muscle region. In panel C, type I (dark stain), IIa (light stain), and IIb-IIc/x (intermediate stain) fibers can be identified based on the myofibrillar actomyosin ATPase histochemical staining procedure [18]. Immunohistochemistry was used to positively identify subpopulations of fibers expressing only the 2B MHC. Panel D shows positive binding (dark) of BF-F3 antibody against 2B MHC. In this manner subpopulations of IIb and IIc/x fibers were identified. Panel E shows positive binding (dark) of the NCL-MHCs antibody against the slow MHC (type I), and panel F shows positive binding (dark) of the A4.74 antibody against 2A MHC. Identification of I and IIa fibers by immunohistochemistry was fully concordant with identification of these fiber types by the myofibrillar actomyosin ATPase histochemical staining procedure.

Northern analysis

Figure 2 shows representative Northern blots for total VEGF mRNA levels examined in (A) resting control, (B) exercised, and (C) electrically stimulated muscles. It is clear that VEGF mRNA levels are elevated over resting after both 1 hour of treadmill exercise and 1 hour of electrical stimulation. This is shown clearly in Figure 3 which gives the quantitative densitometry for VEGF mRNA, normalized to 18S ribosomal RNA. A significant increase in VEGF mRNA over resting control is observed after 1 hour of treadmill running (~3.5-fold, $P = 0.015$), and after 1 hour of electrical stimulation (~3.5-fold, $P = 0.015$). There were no significant differences in the VEGF mRNA levels between exercised and electrically stimulated muscles.

Regional VEGF mRNA signal

Oxidative regions were located in the deep part of the gastrocnemius, and were identified based on the expression of a majority of type I and IIa fibers (~77% of the total fiber number in the regions analyzed). Glycolytic regions were located superficially, and were identified based on the expression of only type IIb and IIc/x fibers.

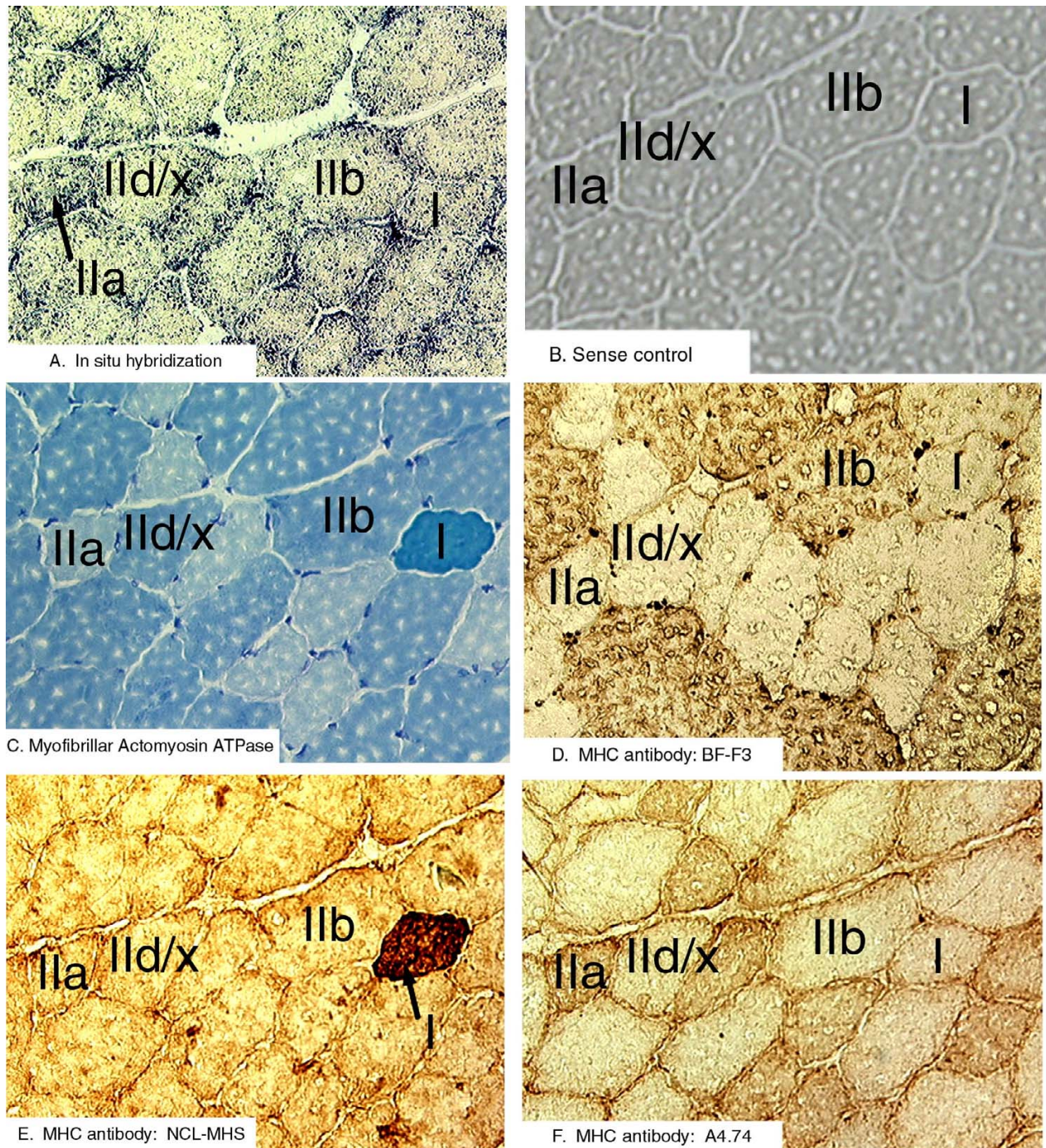


Figure 1

Serial sections of rat gastrocnemius muscle. This is muscle after a 1 hr treadmill run processed through *in situ* hybridization, histochemistry, and immunohistochemistry. (A) Intracellular location (dark stain) of VEGF₁₆₅ mRNA transcripts from *in situ* hybridization with representative type I, IIa, IIb and IIb-IId/x fibers indicated. (B) Sense strand control from *in situ* hybridization which shows no significant non-specific background staining in this protocol. (C) Myofibrillar actomyosin ATPase staining procedure to identify type I (dark stain), type IIa (light stain), and the mixed population of type IIb-IId/x (medium stain) fibers. (D) Positive binding of 2B MHC antibodies (BF-F3) to type IIb fibers is shown as dark stain. (E) Positive binding of slow MHC antibodies (NCL-MHCs) to type I fiber shown as dark stain. (F) Positive binding of 2A MHC antibodies (A4-74) to type IIa fibers shown as dark stain. Magnification by 25× objective in all photos.

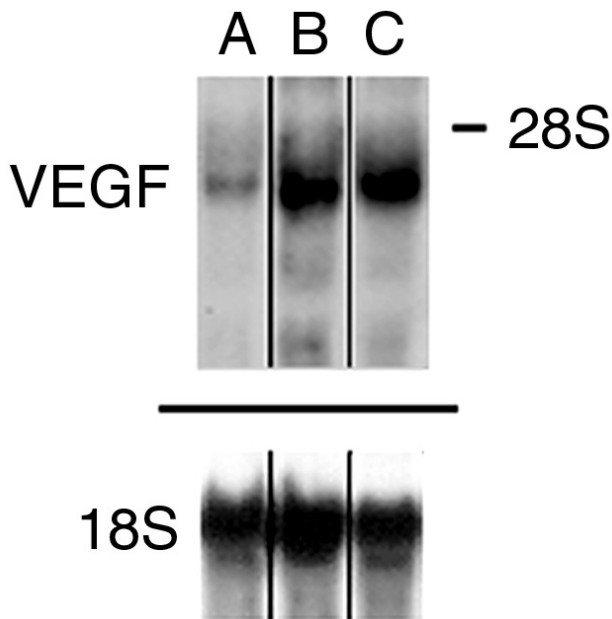


Figure 2
Representative Northern blot for total VEGF mRNA levels. (A) resting, (B) exercised, and (C) electrically stimulated gastrocnemius muscles. Blots were reprobated and normalized to 18S ribosomal RNA (18S).

The heterogeneity of fiber phenotypes in oxidative regions affords the possibility of comparing VEGF mRNA signal between adjacent fibers differing in metabolic capacity. However, data collected in this regard were considered insufficient to test the hypothesis that fiber phenotype is related to VEGF mRNA signal strength. That is, a non-biased test of this hypothesis requires a random sampling of fibers from each phenotypic class in multiple muscle samples. Such fibers must then be clearly identified on serial slide preparations to determine fiber phenotype and VEGF mRNA signal. This was not possible in all muscle samples due to freezing artifact and tissue degradation during (particularly) the *in situ* hybridization process. Regional comparisons are not limited in this way as muscle regions were easily identified on serial sections for measurement of VEGF signal in a random sample of fibers within the region (see Methods).

Data presented in Table 1 show the normalized mean VEGF mRNA signal in oxidative versus glycolytic muscle regions from rats after 1 hour of treadmill exercise, rats after 1 hour of electrical stimulation, and control rat muscle (neither exercise or electrical stimulation). Mean VEGF signal was determined from 27 randomly sampled fibers in each defined region within a single muscle sample (see Methods). By ANOVA, there were significant differences in VEGF mRNA signal strength between functional re-

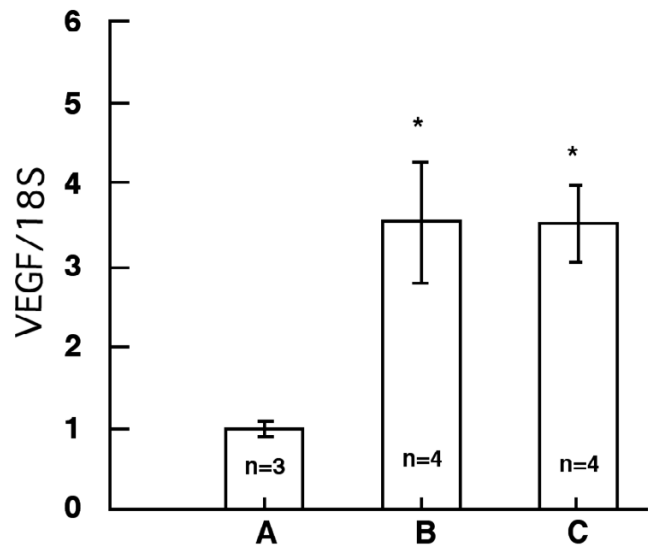


Figure 3
Quantitative densitometry of total VEGF mRNA levels. (A) resting, (B) exercised, and (C) electrically stimulated rat gastrocnemius muscles. Lane loading was normalized by densitometry on 18S ribosomal RNA. *Significantly different from rest-control.

gions ($P = 0.002$) and between protocols (treadmill, electrical stimulation, and control, $P = 0.036$). In treadmill run muscle the glycolytic regional mean was 93% of the oxidative regional mean ($P \leq 0.05$), and in electrically stimulated muscle the glycolytic regional mean was 59% of the oxidative regional mean ($P \leq 0.05$). Significant differences were not apparent in control muscle ($P = 0.463$). *Post-hoc* testing reveals a significantly larger regional difference in VEGF mRNA signal in electrically stimulated versus treadmill run muscle ($P = 0.017$), and no significant difference in this regard between electrically stimulated and control muscle, although the latter comparison reaches a P-value of 0.056. Regional differences were not significantly different between treadmill run and control muscle ($P = 0.912$).

Discussion

Previous studies demonstrate that an acute bout of exercise or electrical stimulation is sufficient to significantly upregulate total VEGF mRNA levels in rat and human skeletal muscle within 1 hour [10,12,15,19]. We have replicated these previous findings by showing (through Northern analysis) that 1 hr. of treadmill running or electrical stimulation induces an approximately 3.5-fold upregulation in VEGF mRNA in rat gastrocnemius muscle (Figure 2 and 3). More specifically, in the present descriptive study, non-radioactive *in situ* hybridization with a VEGF₁₆₅ probe [20] was used to visualize the fiber specific localization of VEGF mRNA transcripts on muscle tissue

Table 1: Normalized¹ mean VEGF₁₆₅ signal from *in situ* hybridization in fibers randomly sampled from oxidative and glycolytic regions of the rat gastrocnemius. Values are given as mean \pm SE.

	Oxidative region		Glycolytic region	
	Number of fibers sampled	VEGF signal (Mean of fibers sampled)	Number of fibers sampled	VEGF signal (Mean of fibers sampled)
Treadmill				
Muscle 1	27	1.00 \pm 0.05	27	0.93 \pm 0.05
Muscle 2	27	1.00 \pm 0.03	27	0.95 \pm 0.02
Muscle 3	27	1.00 \pm 0.03	27	0.92 \pm 0.03
Muscle 4	27	1.00 \pm 0.02	27	0.91 \pm 0.01
	Mean ²	1.00 \pm 0.00		0.93 \pm 0.01*
Electrical				
Muscle 1	27	1.00 \pm 0.04	27	0.76 \pm 0.03
Muscle 2	27	1.00 \pm 0.11	27	0.63 \pm 0.14
Muscle 3	27	1.00 \pm 0.09	27	0.65 \pm 0.06
Muscle 4	27	1.00 \pm 0.69	27	0.31 \pm 0.25
	Mean ²	1.00 \pm 0.00		0.59 \pm 0.1*
Control				
Muscle 1	27	1.00 \pm 0.04	27	0.83 \pm 0.04
Muscle 2	27	1.00 \pm 0.03	27	0.99 \pm 0.05
	Mean ²	1.00 \pm 0.00		0.93 \pm 0.02

¹Normalized within each muscle sample to the mean of the oxidative region. ²ANOVA mean using individual muscles as the unit of analysis (P = 0.002 for regional differences and P = 0.036 for protocol differences). *Significantly different from mean of oxidative region by paired t-test, p \leq 0.05.

cross sections after muscles were stimulated to upregulate VEGF through exercise or electrical stimulation. This technique revealed modest VEGF signal differences between functional regions of the rat gastrocnemius that differed in oxidative capacity, with oxidative muscle regions showing higher signal after both electrical stimulation and treadmill running.

In situ hybridization allows visualization of the intracellular location of mRNA transcripts, but it is not a technique designed to provide a quantitative measure of mRNA signal strength. This is problematic, even for quantitative comparison between muscle regions on the same slide preparation. For example, signal strength can vary across a slide preparation with probe binding success, which depends on the elimination of endogenous RNases, and on tissue permeability to the VEGF probe [21]. Additional error is introduced by freezing artifacts, which are difficult to avoid, and non-specific background signal. To address the latter limitation, we successfully minimized background signal as demonstrated in a control muscle cross-section probed with a sense strand transcript of the VEGF probe (Figure 1B). Error is not necessarily an insurmountable problem so long as it is randomly distributed across muscle slide preparations. On the assumption of random error distribution, our approach was to use the individual muscle as the unit of analysis and to analyze multiple

muscle samples. Fibers were randomly sampled from muscle functional regions to determine a regional mean VEGF signal within each muscle sample, and this signal was normalized to an internal standard to allow for direct comparison between muscles (see Methods). Non-random error (confounding) was also considered, including fiber size differences between regions and the number of nuclei visible in myofibrillar actomyosin ATPase slide preparations between regions (see Figure 1C). While fibers were larger in glycolytic regions, size differences did not relate to VEGF signal. Also, the VEGF probe did not co-localize with muscle nuclei within the fiber (see Figure 1A), another possible source of confounding.

Relevant to the main finding of this study, that VEGF mRNA signal is stronger in deep oxidative regions of the muscle expressing a high proportion of type I and IIa fibers, is the extensive literature describing skeletal muscle capillarity, blood flow, and the angiogenic response to exercise or electrical stimulation [22]. Fibers with an oxidative phenotype have a greater number of surrounding capillaries than glycolytic fibers [23]. This difference in vascular supply is consistent with the metabolic profiles of type I and IIa fibers versus IIb and IIc/x fibers. The former have a high oxidative capacity and high demand for O₂ delivery, while the latter have a lower oxidative capacity and a reduced demand for O₂[17]. Studies of mixed mus-

cle show greater capillarity and/or higher rates of blood flow during exercise in regions composed primarily of oxidative fibers compared to regions with a high proportion of glycolytic fibers [24–28]. Thus, the regional difference observed in VEGF signal may be related to differences in innate fiber phenotype. However, we cannot exclude other possibilities. For example, the difference may be related to differences in metabolic activity between regions during the stimulation protocols, rather than fiber phenotype *per se* (see below), or indeed to regional differences in other VEGF producing cell types e.g., endothelial cells which are well known to produce VEGF [29].

Whether the regional differences in VEGF mRNA demonstrated here underlie either 1) regional differences in capillarity or 2) regional differences in exercise induced angiogenesis cannot be determined from our study. Regarding angiogenesis, our findings are not consistent with previous studies which suggest that capillary growth in response to electrical stimulation is initiated in the vicinity of IIB fibers, not I or IIA fibers [14,22]. However, our findings are consistent with recent findings by Annex et al. [30]. These authors demonstrate that VEGF mRNA upregulation after chronic electrical stimulation is followed by an increase in VEGF protein in the extracellular matrix between fibers, and that the protein increase is highest in innate oxidative versus innate glycolytic muscle. The increase in protein takes place over a 21 day time frame which is much longer than the transient increase in VEGF message demonstrated here, but certainly noteworthy.

We cannot explain why electrical stimulation tends to produce larger differences in VEGF mRNA signal compared to treadmill running. Indeed, treadmill running did not produce a regional difference that was significantly different from the control muscle processed through *in situ* hybridization. This may be related to the small number of control muscles processed, or with fundamental differences between the exercise and electrical stimulation protocols. The running and electrical stimulation protocols differ in the way that motor units are involved during muscle contraction, and thus they may also differ in the pattern of VEGF gene activation between muscle regions. During exercise, slow twitch motor units serving oxidative fibers are recruited first during moderate exercise, and motor units serving glycolytic fibers are recruited later and during more severe exercise [1]. This is in contrast to electrical stimulation of sufficient intensity where all motor units are thought to be activated simultaneously [31]. The larger regional difference in VEGF mRNA signal after electrical stimulation may be due to the fact that this protocol was more metabolically demanding than the treadmill exercise protocol, or indeed because the rat muscle was hypoxic during electrical stimulation. In support of the first possibility, we observed a significant force de-

cline in many of the electrically stimulated muscles i.e., some muscles showed a decline in peak tension development of more than 25% by the end of 1 hr. of stimulation. However, we have no corroborative evidence to support this possibility such as the glycogen content of muscle fibers after exercise or electrical stimulation. In support of the latter possibility, the gastrocnemius muscle may have been hypoxic, despite the fact that all rats were mechanically ventilated during the electrical stimulation protocol. Hypoxia has been demonstrated to upregulate VEGF independent of exercise [10], although in this case we would have expected higher total VEGF levels by Northern analysis in the electrically stimulated muscles, and this was not the case (Figures 2 and 3).

Conclusions

In summary, this descriptive study demonstrates modest regional differences in the mean VEGF mRNA signal in rat gastrocnemius muscles that were induced to upregulate VEGF production by 1 hr. of treadmill running or electrical stimulation. This regional patterning of VEGF mRNA is at least superficially consistent with differences in capillarity and oxidative capacity, although not consistent with the angiogenic process itself which may be preferentially initiated around IIB fibers. Despite some consistencies with morphologic and metabolic observations, it is not possible to conclude that the observed VEGF mRNA differences are physiologically important, or that they are involved in initiating and/or maintaining regional heterogeneity in muscle capillary density. However, the technique of *in situ* hybridization may prove useful to address these questions in the future.

Materials and Methods

Animals

This study was approved by the University of California, San Diego, Animal Subjects Committee. Female Wistar rats were used (age 6–8 weeks, ~200 grams body weight). All animals were housed in cages and allowed standard rat food and water ad libitum prior to study.

Study protocols used to induce upregulation of VEGF mRNA

Both treadmill exercise and muscle electrical stimulation protocols were used to initiate the total VEGF mRNA response in rat skeletal muscle. For the exercise protocol, 6 rats were first familiarized with a rodent treadmill (Omni-pacer model LC-4, Omnitech, Columbus, OH), and then required to run for 1 hour at 20–35 m/min on an incline of 10°. Running speed varied between rats but was maintained for an individual rat near the maximal running speed that could be sustained for a 1 hour period. For rats of this age and body size we have previously shown that a running speed of 20 m/min at 10° incline represents ~55% of maximal oxygen consumption [10].

For the electrical stimulation protocol 6 rats were anesthetized by i.p. injection of sodium pentobarbital. Because anesthetization depresses ventilatory drive, and because hypoxia can also induce VEGF mRNA upregulation, great care was taken in the electrical stimulation protocol to ensure that animals were adequately mechanically ventilated. For each rat, the trachea was intubated and ventilation was maintained at a tidal volume of 2.5 ml, ventilatory frequency of 50 breaths/min, and 1 cm of positive end expiratory pressure. Preliminary work showed this level of ventilation to be sufficient to preserve normal arterial PO₂ and PCO₂. The left gastrocnemius, soleus, and plantaris muscles were surgically isolated and the sciatic nerve, which innervates this muscle complex, was cut and attached to an electrode. The hindlimb was fixed so that no movement of the limb was possible during muscle contraction and the Achilles tendon was attached to a force-displacement transducer (Grass Instruments, Co., Quincy, Mass) to monitor tension development during contraction. Contractions were initiated by train rate impulses, 4–8 volts, 200-ms duration, 1 ms⁻¹ frequency, given at a rate of 2 stimulations (contractions) per second for 1 hour. Signs of muscle fatigue i.e., a decline in tension development below 75% of peak, were evident using this stimulation protocol after 30–60 minutes in approximately 50% of the rats tested. In the remaining rats, tension was well maintained over the course of the stimulation protocol. There was no detectable difference in VEGF mRNA levels by Northern analysis between muscles that fatigued versus muscles that maintained tension development. Six resting rats (non-exercised) were processed as a control for Northern Analyses and *in situ* hybridization. These were the age matched cage mates of those rats run on the treadmill (2 rats per cage). With the exception of the exercise protocol, these rats were handled in an identical manner to the exercised rats.

Tissue collection and processing

After the exercise protocol rats were allowed to rest for 1 hour and then were anesthetized by i.p. injection of sodium pentobarbital. For the 6 rats run on the treadmill, the left gastrocnemius (both heads combined) was removed for RNA isolation, frozen in liquid nitrogen, and stored at -80°C. In these same rats the right gastrocnemius was removed for *in situ* hybridization and fiber type determinations. This tissue was mounted in TBS tissue freezing medium (Triangle Biomedical Sciences, Durham, NC), frozen in isopentane cooled in liquid nitrogen, and stored at -80°C. For the 6 electrically stimulated rats, skeletal muscle was also harvested 1 hour after the completion of the electrical stimulation protocol. Electrical stimulation can be applied to one leg at a time only. In our protocol the left leg is used. Thus, the left gastrocnemius was harvested for RNA isolation and Northern analyses in six rats. An *additional* four rats provided a left gastrocnemius for *in*

situ hybridization, histochemistry, and immunohistochemistry. This minor inconsistency in study design is not expected to affect study results. Six resting rats provided a right gastrocnemius as a control for Northern analysis.

For Northern analysis, 18 muscle samples were initially processed (6 rest control, 6 electrically stimulated, and 6 treadmill run), but some samples were degraded during the RNA isolation leaving 3 rest-control, 4 exercised, and 4 electrically stimulated muscle samples. In order to ascertain regional and fiber type patterns of VEGF mRNA production in those muscles stimulated to upregulate VEGF, 4 each of treadmill exercised and electrically stimulated muscle samples, and 2 control muscles, were successfully processed through *in situ* hybridization.

RNA isolation and Northern analyses

Total cellular RNA was isolated from each gastrocnemius muscle by the method of Chomczynski and Sacchi [32]. RNA preparations were quantified by absorbance at 260 nm and RNA intactness and integrity assessed by ethidium bromide staining after separation by electrophoresis in a 6.6% formaldehyde-1% agarose gel. Fractionated RNA was transferred by Northern blot to Zeta-probe membrane (Bio-Rad, Hercules, CA). Following transfer, RNA was cross-linked to the membrane by ultraviolet irradiation for 1 min and stored at 4°C. The blots were then probed with oligolabeled [α -³²P] deoxycytidine triphosphate cDNA probes which had a specific activity > 1 × 10⁹ disintegrations.min-1.μgDNA-1(11). The rat VEGF probe is a 0.9 kb cDNA Pst I/Sma I insert cloned into a pBluescript II KS+ vector [33]. Prehybridization and hybridization were performed in 50% formamide, 5×SSC (20×SSC is 0.3 M sodium chloride, 0.3 < sodium citrate), 10× Denhardt's solution (50× Denhardt's solution is 2% Ficoll, 2% polyvinyl pyrrolidone, 2% Bovine Serum Albumin Factor V), 50 mM sodium phosphate (pH 7.0), 1% sodium dodecyl sulfate (SDS), and 250 μg/ml salmon sperm DNA at 42°C. Blots were washed with 2×SSC and 0.1% SDS at room temperature and 0.1% SSC and 0.1% SDS at 65°C. Blots were exposed to XAR-5 X-ray film (Eastman Kodak, New Haven, CT) for 2–3 days by use of a Cronex Lighting Plus screen at -80°C. Autoradiographs were quantitated by densitometry within the linear range of signals and normalized to ribosomal 18S RNA levels.

In situ hybridization

Frozen gastrocnemius muscle samples were cryo-sectioned in a Reichert Jung Cryocut 1800 cryostat (Cambridge Instruments, Buffalo, NY) at -20°C to 10 μm and mounted in cross-section on Fisherbrand Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Slides were immediately processed through the *in situ* hybridization protocol of Braissant and Wahli [20] to detect VEGF mRNA transcripts on tissue sections. After tissue fix-

ation for 20 minutes in 4% paraformaldehyde in diethyl pyrocarbonate (DEPC) treated phosphate buffered saline (PBS), sections were incubated for 2 × 15 minutes in PBS containing 0.1% active DEPC, and equilibrated for 15 min in 5×SSC. The sections were then prehybridized for 2 hr at 58°C in the hybridization mix (50% formamide, 5×SSC, salmon sperm DNA 40 µg/µl; 500 µl on each section). The hybridization probes (see below) were denatured for 5 min at 80°C and added to the hybridization mix (400 ng/ml). The hybridization reaction was carried out overnight at 58°C with 50 µl of hybridization mix on each section. During hybridization, sections were kept in a box saturated with 5×SSC, were covered by a rectangle of Parafilm, and were sealed with DPX Mountant (Fluka Chemie, Neu-Ulm, Switzerland) to prevent drying. After hybridization, sections were washed for 30 min in 2×SSC (room temperature), 1 hour in 2×SSC (65°C), 1 hr in 0.1×SSC (65°C), and equilibrated for 5 min in Buffer 1 (Tris-HCl 100 mM and NaCl 150 mM, pH 7.5). Sections were then incubated for 2 hours at room temperature with alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer Mannheim, Indianapolis, IN) diluted 1:5000 in Buffer 1 containing 0.5% Blocking reagent (Boehringer Mannheim). Excess antibody was removed by two 15 min washes in Buffer 1, and the sections were equilibrated for 5 min in Buffer 2 (Tris-HCl 100 mM, NaCl 100 mM, and MgCl₂ 50 mM, pH 9.5). Color development was performed at room temperature overnight in Buffer 2 containing NBT and BCIP (Boehringer Mannheim). Staining was stopped by a 10 min wash in Tris/EDTA (10/1 mM, pH 8.0), and non-specific staining was removed by 1 hour in 95% EtOH. Sections were rehydrated in deionized water for 15 min to remove precipitated Tris and then dehydrated through successive ethanol baths (70, 95, 100%) and Hemo-De (Fisher Scientific) (2 × 15 min each) and mounted in Permount (Fisher Scientific).

Hybridization probes were derived from a ~600-bp cDNA that contains the entire coding region of the human VEGF₁₆₅[8] subcloned into the pBluescript II KS+ vector (Stratagene, La Jolla, CA). The final riboprobe was the same size (~600-bp) as the cDNA. Constructs in this vector were linearized at appropriate restriction sites to allow the synthesis of digoxigenin-UTP labeled complementary RNA in the antisense or sense orientation (using T3 or T7 RNA polymerase, respectively; Boehringer, Indianapolis, IN). The riboprobes synthesized in the "sense" orientation served as background control.

Determination of fiber types

Fiber types were determined in serial cross sections. Two methods were used: (1) The *histochemical* method of Ogilvie and Feedback [18], based on staining for myofibrillar actomyosin ATPase, and (2) *immunohistochemistry* based on the reaction of specific antibodies for myosin heavy

chain (MHC) isoforms [34,35]. In the histochemical staining procedure, sections were pre-incubated in (0.025 M potassium acetate, 9 mM calcium chloride-dihydrate, pH to 4.5 with glacial acetic acid) for 8 minutes, rinsed 3× two min/each in (0.1 M Trizma base (Sigma), 0.018 M calcium chloride-dihydrate), and incubated for 25 minutes in (0.154 M ATP-disodium salt, 0.053 M Glycine, 0.029 M calcium chloride, 0.065 M sodium chloride, 0.048 M sodium hydroxide, pH 9.4). Following incubation sections were rinsed 3× in 0.014 M calcium chloride-dihydrate, stained for 1 min in Toluidine blue, rinsed for 5 sec in deionized water, and dehydrated with 5 dips in 95% ethanol, 5 dips in 2 changes of 100% ethanol, and 2 changes of Hemo-De (Fisher) for 5 min each. Sections were mounted with Permount (Fisher). This method allows for the clear determination of type I and IIa fibers, but distinguishing IIb from IIc/x subpopulations is difficult and subjective as both stain with nearly the same intensity. For this reason immunohistochemistry was also used on one muscle sample to clearly identify IIb fibers based on specificity of the 2B MHC antibody. For immunohistochemistry, mouse polyclonal and monoclonal antibodies specific for slow (Novocastra, NCL-MHCs), 2A (Blau, A4.74), and 2B (DSM, BF-F3) MHC isoforms were used. Serial sections were incubated overnight with primary antibodies diluted (NCL-MHCs, 1:100; and BF-F3, A4.74, 1:1) in a 0.2 M potassium phosphate solution (PPS). After washing 3× with PPS, sections were incubated for 3 hr at room temperature with either peroxidase-conjugated goat anti-mouse immunoglobins (for NCL-MHCs, and A4.74) or biotinylated rabbit antimouse IgM (for BF-F3) (DAKO A/S, Glostrup, Denmark). The latter was also incubated for 1 hour with streptavidin-HRP (NEN Life Science Products, Boston, MA) diluted 1:100 in PPS. Sections were again washed 3× in PPS and color was evolved by incubating slides in the dark between 2–10 min in a 3,3'-diaminobenzidine (DAB) solution (Vector Laboratories Inc., Burlingame, CA). Sections were rinsed in deionized water, dehydrated in successive ethanol baths, cleared with Hemo-De (Fisher), and mounted with Permount (Fisher).

Quantification of the regional VEGF mRNA signal from *in situ* hybridization

Regional VEGF mRNA signal within an individual muscle fiber was quantified by densitometry from microscope images obtained by a Sony 3ccd color video camera (Sony Corporation, Japan) attached to a Jenalumar optical microscope (Jenoptik JENA, Germany), and processed by the Sigma Scan Pro imaging software (SPSS Inc., Chicago, IL). Optical density measurements were made on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available from the internet by anonymous FTP from [zip-py.nimh.nih.gov]). This program can calculate mean grey-

scale value within the cross sectional area of a single muscle fiber.

Unfortunately, measured grey-scale values give arbitrary units. Within a given microscopic field of view, an optical density measurement depends both on the strength of the VEGF mRNA signal, and also on the amount of light transmitted through the tissue sample (which in turn depends on the degree of freezing artifact in muscle preparation and many other factors). Uncontrolled factors affecting light transmission imply that optical density comparisons between different slide preparations are not valid. In order to aggregate data from different slides, it was necessary to normalize all VEGF mRNA optical density signals to an internal standard. For example, to describe the difference between an oxidative and glycolytic muscle region, the optical density value of each individual fiber was normalized to the mean of all fibers within the oxidative region (which was arbitrarily set to a value of 1.0) (see Table 1).

A random sampling strategy was used to obtain mean optical density values for different muscle regions identified on histochemically stained sections. Oxidative regions were located in the deep part of the gastrocnemius and expressed a majority of type I and IIa fibers (~77%), while glycolytic regions were located superficially, and expressed only type IIb and IIc/x fibers. For each muscle, a microscopic field of view was located in a region which contained typically between 100–200 fibers in cross section at 125× magnification. The same field of view was then located on a serial section processed through *in situ* hybridization. Twenty seven fibers total (three fibers from each of 9 equal quadrats) were randomly selected from the region on the *in situ* slide preparation and densitometric measurements of the VEGF mRNA signal within fibers were made.

Analyses and statistics

Analysis of variance was used to test for differences in VEGF mRNA levels from Northern analysis between muscle samples from exercised (n = 4) and electrically stimulated rats (n = 4). ANOVA, using the individual muscle sample as the unit of analysis, was also used to test for the main effects of functional region (oxidative vs. glycolytic) and protocol (treadmill running, electrical stimulation, control) on VEGF mRNA signal. *Post-hoc* testing, using Fisher's Least Square Difference correction for multiple comparisons was used to test for differences between protocols in the regional difference of VEGF mRNA signal. Paired t-tests were used to compare regional means within a given protocol. All statistical analyses were performed using Systat Version 5.2 (Systat Inc., Evanston, IL). Statistical significance was set at $p \leq 0.05$ for all tests.

Authors' contributions

Author 1 (TB) participated in the conception, design, and coordination of the study, carried out the animal protocols, tissue processing through *in situ* hybridization, histochemistry, and immunohistochemistry, microscopic quantification of VEGF signal, and drafted the manuscript. Author 2 (TG) did the Northern analysis. Author 3 (ZF) participated in the immunohistochemistry and *in situ* hybridization. Author 4 (EB) participated in the immunohistochemistry and *in situ* hybridization. Author 5 (5) participated in the *in situ* hybridization. Author 6 (OMC) participated in the design and coordination of tissue processing and histochemistry. Author 7 (PW) participated in the conception, design, and coordination of the study.

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