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Regulation of VEGF and bFGF mRNA expression and other proliferative compounds in skeletal muscle cells

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Abstract

The role of muscle contraction, prostanoids, nitric oxide and adenosine in the regulation of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and endothelial cell proliferative compounds in skeletal muscle cell cultures was examined. VEGF and bFGF mRNA, protein release as well as the proliferative effect of extracellular medium was determined in non-stimulated and electro-stimulated rat and human skeletal muscle cells. In rat skeletal muscle cells these aspects were also determined after treatment with inhibitors and/or donors of nitric oxide (NO), prostanoids and adenosine. Electro-stimulation caused an elevation in the VEGF and bFGF mRNA levels of rat muscle cells by 33% and 43% ($P < 0.05$), respectively, and in human muscle cells VEGF mRNA was elevated by 24%. Medium from electro-stimulated human, but not rat muscle cells induced a 126% higher ($P < 0.05$) endothelial cell proliferation than medium from non-stimulated cells. Cyclooxygenase inhibition of rat muscle cells induced a 172% increase ($P < 0.05$) in VEGF mRNA and a 104% increase in the basal VEGF release. Treatment with the NO donor SNAP (0.5 μM) decreased ($P < 0.05$) VEGF and bFGF mRNA by 42 and 38%, respectively. Medium from SNAP treated muscle cells induced a 45% lower ($P < 0.05$) proliferation of endothelial cells than control medium. Adenosine enhanced the basal VEGF release from muscle cells by 75% compared to control. The present data demonstrate that contractile activity, NO, adenosine and products of cyclooxygenase regulate the expression of VEGF and bFGF mRNA in skeletal muscle cells and that contractile activity and NO regulate endothelial cell proliferative compounds in muscle extracellular fluid.

Introduction

Although it is well known that regular contractile activity induces an increased capillarisation in skeletal muscle [1], little is known about the regulation of angiogenesis in this tissue. Angiogenesis in skeletal muscle in response to exercise has been attributed to increases in blood flow and accompanying capillary shear stress and/or wall tension and due to the contractile activity itself. All of these stimuli are likely to be important, but very few studies have investigated the isolated role of muscle contraction on growth factor expression and release, and on endothelial cell proliferation.

Several growth factors may be involved in the angiogenic process, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Both have been shown to be important angiogenic factors in many different kinds of tissues [2, 3]. Experiments in laboratory animals show that muscle contraction induces an increase in VEGF and bFGF mRNA in muscle tissue [4] and studies on humans have shown increases in VEGF in the muscle interstitial fluid during contraction [5], indicating that these growth factors also may have a role in angiogenesis in the skeletal muscle. However, as measurements of VEGF and bFGF expression in most previous studies have been performed in whole muscle homogenates containing several cell types it is not clear in which cells alterations in levels of VEGF and bFGF mRNA in response to muscle contraction occur. Contracting muscle cells may be a source of VEGF and/or bFGF in skeletal muscle and the release of these growth factors can cause endothelial cell proliferation by binding to their receptors which in the case of VEGF are located exclusively on endothelial cells [6, 7]. The possibility of muscle cells being an important source of endothelial growth factors is supported by findings of Kanno et al. [8] who reported a contraction-induced increase in the expression of VEGF in the C2C12 muscle cell line. The muscle contraction-induced regulation of VEGF and bFGF expression and protein release has however, not previously been examined in rat or human primary skeletal muscle cells.

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Compounds formed in skeletal muscle [9–11] such as PGI_2 (prostacyclin), TXA_2 (thromboxane A_2), $PF_{2\alpha}$ (Prostaglandin $F_{2\alpha}$), PE_2 (Prostaglandin E_2), nitric oxide (NO) and adenosine have been shown to regulate angiogenesis and/or the expression of VEGF and bFGF in various cells and tissues [12–15]. However, findings in the literature diverge with regard to whether these compounds up-regulate or down-regulate VEGF and bFGF in different tissues, and the role of these compounds in skeletal muscle capillary growth remains unresolved. Prostacyclin has been shown both to decrease [16] and increase [17] VEGF mRNA and the same has been seen with prostaglandins [18, 19]. Inhibition of cyclooxygenase (COX), which prevents the production of prostaglandins, prostacyclin and thromboxane A_2, was found to attenuate electrical stimulation-induced capillary growth in rat skeletal muscle [20].

NO donors in rat skeletal muscle were shown to increase VEGF mRNA, but not bFGF mRNA [21] whereas NOS inhibition in human skeletal muscle has been found to have no effect on either basal VEGF or bFGF mRNA expression [22]. In vitro, NO inhibited a bFGF stimulated-endothelial cell proliferation [23], but NO has also been shown to increase angiogenesis [24].

An adenosine receptor agonist induced VEGF mRNA in U-937 cells [25] and smooth muscle cells [26], however, the role of adenosine in growth factor-mediated angiogenesis has not been studied in skeletal muscle.

The purpose of the present study was to elucidate (a) the role of muscle contraction, NO, prostanoids and adenosine on the expression and release of VEGF and bFGF in skeletal muscle cells in culture and (b) the effect of extracellular medium from skeletal muscle cells on endothelial cell proliferation. Primary skeletal muscle cell cultures obtained from rat tissue were used as the primary experimental model, however, the effect of muscle contraction on VEGF and bFGF expression and on endothelial cell proliferation was also examined with primary human skeletal muscle cells. Endothelial cell proliferation was assessed using human umbilical vein endothelial cells (HUVECs).

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), horse serum (HS), Dulbecco's phosphate buffered saline (DPBS), matrigel, penstrep (penicillin [10,000 U/ml], streptomycin [10,000 U/ml]), trypsin and culture dishes were all from Life Technologies. Human Umbilical Vein Endothelial Cells (HUVECs) in the primary culture stage and medium 200 with Low Serum Growth Supplement (LSGS) containing foetal bovine serum, fibroblast growth factor, heparin and epidermal growth factor (Cascade Biologics Inc, Portland, Oregon) were obtained from Cytotech (Denmark). Collagenase (type II), DNase, trypsin/EDTA solution, glucose, indomethacin (an inhibitor of cyclooxygenase), N^G-nitro-L-arginine (L-NA) (inhibitor of nitric oxide synthase (NOS)), *S*-nitroso-*N*-acetyl-D, L-penicillamine (SNAP) (an NO analog), α,β-methyleneadenosine 5′-diphosphate (AOPCP) (an inhibitor of ecto 5′-nucleotidase), adenosine, thromboxane B_2 (TXB) (Thromboxane A_2 metabolite), prostaglandin $F_{2\alpha}$ ($PF_{2\alpha}$) and prostaglandin E_2 (PE_2) were all products from Sigma, Missouri. Ilomedin (iloprost) (prostacyclin (PGI_2) donor) was from Noerrebro Pharmacy, Denmark. TriReagent from the Molecular Research Center (Ohio).

Primary skeletal muscle cell cultures

In each experiment, one Wistar male rat (M&B, Denmark) weighing 100 g was anaesthetised with 0.1 ml sodium pentobarbital (50 mg/ml). Carefully the muscle fascia was removed and soleus, gastrocnemius and quadriceps femoris were removed and placed on ice in DPBS (Gibco, Grand Island, New York) with 1% glucose. The muscle tissue was minced into small pieces with scissors and then digested with 0.2% collagenase II in DMEM (Gibco) containing 1% penstrep, for 1.5 h at 37 °C with rotation. After centrifugation at 200 × *g* for 15 min pellet was incubated with rotation in a solution of 0.2% collagenase, 0.01% DNAse and 0.25% trypsin in DMEM containing 1% penstrep for 30 min at 37 °C. The cells were suspended in primary growth medium (PGM) (DMEM supplemented with 1% penstrep, HS (10%) and FCS (10%), Gibco), counted and seeded out onto 35 mm dishes (Nunc, Denmark) coated with 1% matrigel and incubated at 8% CO_2 and 37 °C. After 4 days PGM was changed to primary fusion medium (FM) (DMEM supplemented with L-glutamine [2 mM] and HS (10%)) and after two additional days the primary skeletal muscle cells were ready for experiments. All treatment of animals complied with the European Convention for the Protection of Vertebrate Animals Used for Experimental or other Scientific Purposes (Council of Europe No. 123, Strasbourg, France, 1985).

Isolation and growth of satellite cells from human skeletal muscle

Two biopsies of approximately 100 mg each were obtained from the middle portion of the vastus lateralis muscle using the percutaneous needle biopsy technique with suction [27]. The subjects were healthy men between 19 and 24 years who were given information of the experimental procedure and the potential risks. The subjects gave their informed consent prior to participation in the experiment. The study conformed with the guidelines laid down in the Declaration of Helsinki and was approved by the Ethical Committee of Copenhagen and Frederiksberg, Denmark.

Muscle tissue was minced and then enzymatically dissociated for 45 min by three successive treatments with collagenase, trypsin and DNase in DPBS with 1% glucose. Cells were dissolved in growth medium (DMEM supplemented with 2% FCS (vol/vol) and pen/strep), filtered through a 70 μM steril filter and seeded out on 100 mm gelatin coated cell dishes. After 1 and 3 days of incubation at 37 °C and 8% CO_2/92% air, the medium was replaced and on day 4, the medium was changed to growth medium without pen/strep. Approximately 8 days after seeding out,

the cells were subcultured just prior to reaching confluence and grown on 35 mm matrigel coated cell dishes. Approximately 4 days after sub-culturing the cells had fused and were used for experiments.

Electrical stimulations of rat muscle cells

Before the experiments, the cell medium was changed to serum free medium (SFM)(DMEM with 0.1% BSA) and the cells were incubated for 12 h. Muscle cell cultures were electrically stimulated according to previously described procedures [28]. In brief, the cells were stimulated in an incubator for 2 h at 2 V and a frequency of 50 Hz. The stimuli consisted of 0.5 s trains with 0.5 s pauses between the trains and 1 ms pulse width. At 0, 10 and 24 h after stimulation, cell medium was collected and cells were lysed by TRI reagent prior to collection. All samples were stored at −80 °C.

Experiments with prostanoids, NO and adenosine

Before the experiments, the cell medium was changed to SFM and the cells were incubated for 12 h. In one set of experiments, SFM containing the various pharmacological compounds was added to rat muscle cells and the cells were incubated in this medium for 24 or 48 h. In other experiments, extracellular medium from muscle cells that had been incubated with the pharmacological compounds was transferred to endothelial cells for determination of proliferative effect. The skeletal muscle cells were lysed with TRI reagent and samples were stored at −80 °C until further analysis.

To investigate the effect of prostanoids, indomethacin (an inhibitor of cyclooxygenase) was used at a concentration of 0.3 mM, iloprost (a prostacyclin analog) at 0.29 mM, thromboxane B_2 (TXB) (Thromboxane A_2 metabolite) at 1 μM, prostaglandin $F_{2\alpha}$ ($PF_{2\alpha}$) at 1 μM and prostaglandin E_2 (PE_2) at 1 μM in the medium. The nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine (L-NA), was used at a concentration of 1 mM and the NO donor SNAP, at 0.5 μM. Adenosine formation was inhibited with 50 μM AOPCP and adenosine was added at a concentration of 0.2 mM. Inhibitors and donors were incubated separately with the muscle cells, and in other experiments inhibitors were incubated in combination with a donor in order to verify if these donors were involved in a possible inhibitor-induced effect. Because ethanol was used as a solvent for indomethacin, TXB, $PF_{2\alpha}$, PE_2 and SNAP, controls with the same amount of ethanol (11 μl/ml SFM) were performed in each experiment. When compared to control cells, incubation of cells with ethanol was without effect ($P > 0.05$) on the parameters measured in the muscle and endothelial cells.

Measurement of endothelial cell proliferation

HUVECs were cultured on 0.1% gelatin-coated 35 mm plastic dishes in medium 200. Endothelial cells were identified morphologically under microscope by their formation of confluent monolayers having a cobblestone shape. HUVEC cultures reached 80% confluence in 5–6 days and were subcultured by using a trypsin/EDTA solution. HUVECs supplemented in medium 200 were grown in 96-well plates for 24 h before replacing the medium with medium from muscle cells, SFM (control medium) and medium 200 (positive control). After an additional 24 h of incubation, bromodeoxyuridine (BrdU) was added and incubated for 12 h. Incorporation of BrdU into the DNA was then detected by an immunoassay (Roche, Mannheim, Germany) according to the protocol of manufacturer. The positive control induced in all experiments the highest level of proliferation indicating that endothelial cell growth was not restricted by contact inhibition when measuring the proliferative effect of cell culture medium.

Measurement of VEGF and bFGF mRNA

Northern blot analysis was carried out to determine the level of VEGF and bFGF mRNA in the muscle cells. Total RNA was extracted essentially as described by Chomczynski and Sacchi [81]. Shortly, phases were separated by bromochloropropane, RNA precipitated by isopropanol and, after wash with 75% ethanol, the RNA was solubilised in water. These RNA preparations were quantitated by absorbance at 260 nm. One microgram of RNA was separated on a denaturing formaldehyde agarose gel and transferred by northern blotting to a positively charged nylon membrane (Appligene, France). After transfer, the blots were probed with [α-^{32}P] dATP cDNA probes. The probes were 251 bp human VEGF, 378 bp human bFGF, 272 bp rat VEGF and 317 bp rat bFGF PCR products, respectively, cloned by blunt-end cloning into the *Sma*I site of the pBlueScriptII SK(+) vector [29]. Primer sequences are shown in Table 1. Hybridisation was performed as described by Higginson et al. [30]. Blots were exposed on a phosphor screen and quantitated using a Molecular Imager FX (Bio-Rad, Denmark) to measure the mRNA levels. Each blot was subsequently reprobed (after prior probes were removed) with a

Table 1. Primers used for northern blot.

Target mRNA	Sequence (5′ to 3′)	Product size	Plasmid ID
Human VEGF sense	GGGCCTCCGAAACCATGAAC		
Human VEGF antisense	GGGCACACAGGATGGCTTGA	251 bp	pCM190
Human bFGF sense	ATTGCATCTGCTGTTACCCA		
Human bFGF antisense	TGCTAGGCTGCTGCTGTATG	378 bp	pCM194
Rat VEGF sense	CTTTCTGCTCTCTTGGGTGC		
Rat VEGF antisense	ACTCCAGGGCTTCATCATTG	272 bp	pCM196
Rat bFGF sense	AAGGATCCCAAGCGGCTCTA		
Rat bFGF antisense	TGCCCAGTTCGTTTCAGTGC	317 bp	pCM198

probe specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and this signal was used to normalise the target. The amount of GAPDH mRNA, measured by obtained counts, in muscle cells obtained at different time points after electro-stimulation showed no significant dependence on time or electrical stimulation ($P > 0.05$; data not shown). GAPDH mRNA levels were not altered ($P > 0.05$) by prostanoid, NO or adenosine donors or inhibitors in the muscle cells. The above results confirmed that the GAPDH mRNA level is sufficiently constitutive to be used as an internal control under these conditions.

Measurement of VEGF and bFGF protein in extracellular medium

Medium collected from muscle cells was centrifuged at $3700 \times g$ and the supernatant was analysed for VEGF or bFGF protein by the appropriate (human or mouse/rat) Quantikine ELISA kits (R&D System) according to the protocol of manufacturer.

Stability of mRNA

In one cell experiment, transcription was inhibited by addition of Actinomycin-D at a concentration of 5.3 μM. Actinomycin-D was added 24 h after electrical stimulation or indomethacin treatment and incubated for 0, 2, 4 or 6 h to evaluate the stability of VEGF and bFGF mRNA. Half-lives of mRNA were calculated by drawing the best-fit linear curve on a log-linear plot of percentage of mRNA remaining vs. time in actinomycin-D-treated cells.

Statistical analyses

Before the statistical analysis were performed, all data were logarithmic transformed to obtain normal distribution of data. Data from electro-stimulated and non-stimulated control muscle cells were expressed as fold change relative to the corresponding control 0 h sample, which was set to 1. Two-way ANOVA was used to evaluate effect of time and electrical stimulation. Values from cells treated with inhibitors were expressed as fold change relative to control (SFM treated cells) values, which was set to 1. Effect of inhibitors on the muscle cells was determined by a one-way ANOVA. When significant changes were found, a Student–Newman–Keuls method for multiple comparisons was used to locate differences. A value of $P < 0.05$ was accepted as statistically significant.

Results

Regulation of VEGF and bFGF mRNA and protein in rat skeletal muscle

Effect of electro-stimulation on VEGF and bFGF mRNA and protein in rat skeletal muscle cells

VEGF mRNA and protein. VEGF mRNA levels were 33% higher ($P < 0.05$) at 24 h than at 0 h after electro-stimulation and the level at 24 h was higher ($P < 0.05$) in electro-stimulated cells than in non-stimulated cells (Figure 1a). More than one band was observed (approximately 4.4, 3.7 and 1.7 kb determined by comparing to position of 28S and 18S ribosomal RNA) most likely corresponding to the $VEGF_{188}$, $VEGF_{164}$ and $VEGF_{120}$ splice variants [31, 32]. Quantification was performed on bands with the highest

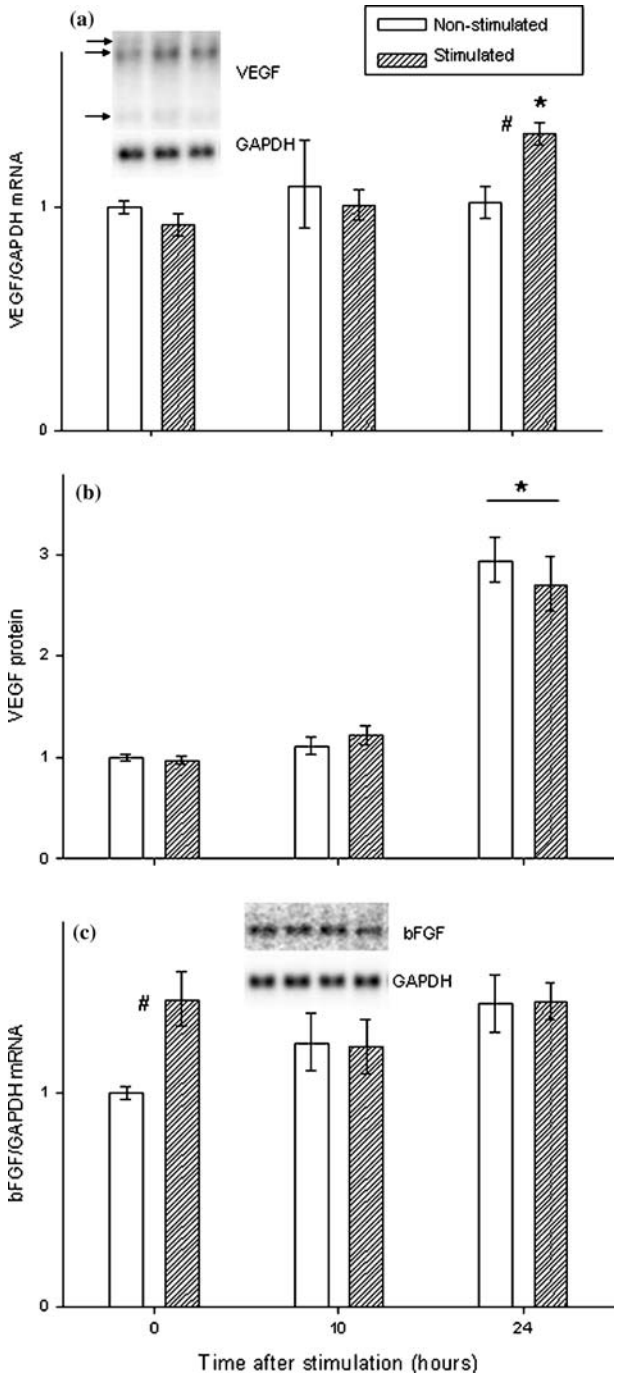

Figure 1. Effect of time and electro-stimulation on VEGF and bFGF mRNA and extracellular VEGF protein in rat skeletal muscle cells. 0, 10 or 24 h after 2 h of either non-stimulation (white bars) or electro-stimulation (grey) primary rat skeletal muscle cells were collected ($n = 27$, 6 and 30, from 9, 3 and 9 independent experiments, respectively). Cells were analysed for content of (a) VEGF mRNA (b) VEGF protein in medium or (c) bFGF mRNA. Values are relative to non-stimulated at 0 h. Insert: representative bands of VEGF and bFGF mRNA and GAPDH obtained by northern blot. $^{*}P < 0.05$ vs 0 h, $^{\#}P < 0.05$ non-stimulated vs stimulated.

intensity ($VEGF_{188}$ and $VEGF_{164}$) and only these are shown in the remaining figures. Changes in intensity were equally distributed between splice variants.

The VEGF protein content in the different experiments ranged from 100 to 3500 pg/ml, with the highest values ($P < 0.05$) observed at 24 h after stimulation or control period. The concentration of VEGF protein in medium obtained from electro-stimulated muscle cells was not different ($P > 0.05$) from that in medium from control cells at any time point (0, 10 or 24 h) after stimulation or control period (Figure 1b).

bFGF mRNA and protein. bFGF mRNA content was 43% higher ($P < 0.05$) in electro-stimulated cells than in control cells at 0 h after stimulation with no differences ($P > 0.05$) after 10 or 24 h (Figure 1c).

The concentration of bFGF protein in medium from muscle cells was below the level of detection (~10 pg/ml) of the assay. This was observed for bFGF measurements under all conditions.

Effect of indomethacin and prostanoid donors on VEGF and bFGF mRNA and protein in rat skeletal muscle cells

VEGF mRNA and protein. Incubation of muscle cells with indomethacin resulted in 170% higher ($P < 0.05$) VEGF mRNA levels than in control cells (Figure 2). Addition of iloprost in combination with indomethacin resulted in a similar ($P > 0.05$) VEGF mRNA level as with indomethacin alone. Addition of either $PF_{2\alpha}$, PE_2 or TXB in combination with indomethacin did not alter ($P > 0.05$) the VEGF mRNA levels from that of control (Figure 2a). Incubation with iloprost, TXB, $PF_{2\alpha}$, or PE_2 alone did not have an effect on VEGF mRNA levels (Table 2).

The concentration of VEGF protein in the muscle extracellular medium was 104% and 99% higher ($P < 0.05$) after incubation with indomethacin or indomethacin in combination with iloprost, respectively (Figure 2b). The VEGF protein concentration was not altered with simultaneous addition of indomethacin and either $PF_{2\alpha}$, PE_2 or TXB ($P > 0.05$) (Figure 2b). Incubation with iloprost, $PF_{2\alpha}$, PE_2 or TXB alone induced no alterations in VEGF release (Table 2).

bFGF mRNA. Muscle cells treated with indomethacin showed a non-significant change of 17% in bFGF mRNA compared to untreated cells. Also, bFGF mRNA content did not differ ($P > 0.05$) between control cells and muscle cells treated with either iloprost, $PF_{2\alpha}$, PE_2 or TXB (Table 2).

Effect of L-NA and SNAP on VEGF and bFGF mRNA and protein in rat skeletal muscle cells

VEGF mRNA and protein. Addition of L-NA to muscle cells did not affect the VEGF mRNA content ($P > 0.05$) whereas addition of the NO donor SNAP resulted in a 42% lower ($P < 0.05$) amount of VEGF mRNA compared to control muscle cells (Figure 3a).

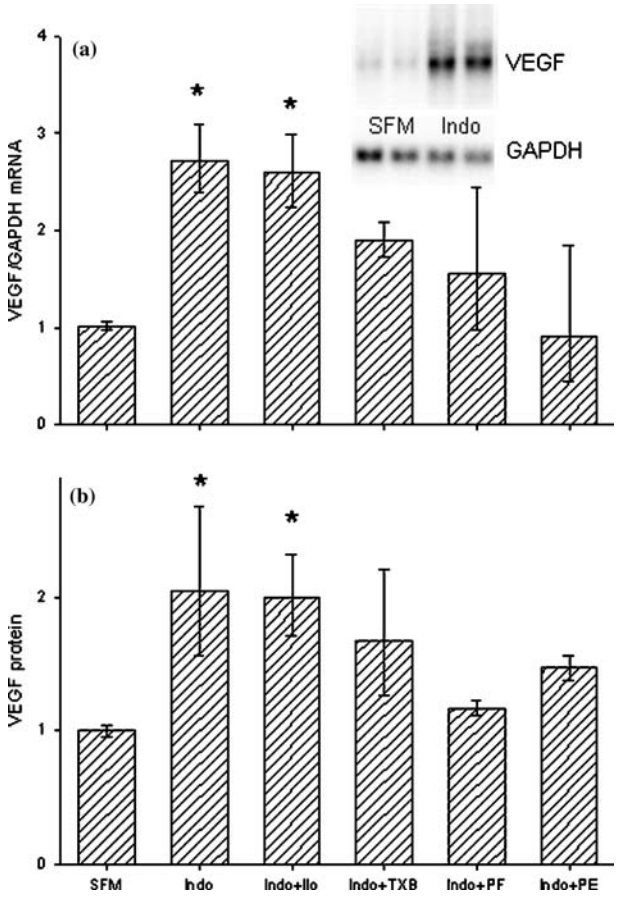

Figure 2. Effect of indomethacin and prostanoid donors on VEGF mRNA and extracellular VEGF protein in skeletal muscle cells. Primary muscle cells were incubated for 24 h with serum free medium (SFM; $n = 52$) or with 0.3 mM indomethacin (an inhibitor of cyclooxygenase) (Indo; $n = 52$) to examine the effect of prostanoids. To verify if some important prostanoids were involved in the inhibitor-induced effect, donors were incubated in combination with indomethacin; 0.3 mM indomethacin + 0.29 mM iloprost (Indo + ilo; $n = 13$), 0.3 mM indomethacin + 1 μM Thromboxane B_2 (Indo + TXB; n = 9), 0.3 mM indomethacin + 1 μM Prostaglandin $F_{2\alpha}$ (Indo + PF; $n = 5$) or 0.3 mM indomethacin + 1 μM Prostaglandin E_2 (Indo + PE; $n = 5$). Cells were analysed for (a) VEGF mRNA and (b) VEGF protein in surrounding medium. Values are relative to SFM. Insert: representative bands of VEGF and GAPDH mRNA obtained by Northern blot. Results are obtained from two (Indo + PF and Indo + PE), three (Indo + TXB), four (Indo + Ilo) or eight (SFM and Indo) independent experiments. *$P < 0.05$ vs SFM.

The VEGF protein concentration in the extracellular medium from muscle cells was not altered ($P > 0.05$) by incubation of the cells with either L-NA or SNAP (Table 3).

bFGF mRNA. No differences in bFGF mRNA levels ($P > 0.05$) were observed in skeletal muscle cells by incubation with L-NA (Figure 3b). Addition of SNAP to the muscle cells caused a 38% lower ($P < 0.05$) amount of bFGF mRNA compared to control muscle cells (Figure 3b).

Effect of AOPCP and adenosine on VEGF and bFGF mRNA and protein in rat skeletal muscle cells

VEGF mRNA and protein. Addition of either AOPCP or adenosine to skeletal muscle cells did not affect VEGF

Table 2. Effect prostanoids on VEGF and bFGF mRNA and protein in rat skeletal muscle cells.

Treatment	Measurement	Relative to control (=100)	SE+ and SE−	n
Iloprost	VEGF mRNA	136	7–7	13
	VEGF protein	182	20–18	14
	bFGF mRNA	106	14–12	14
TXB	VEGF mRNA	88	14–12	9
	VEGF protein	151	25–22	14
	bFGF mRNA	123	27–22	13
PF	VEGF mRNA	101	17–15	5
	VEGF protein	85	16–13	10
	bFGF mRNA	127	9–8	5
PE	VEGF mRNA	107	15–13	5
	VEGF protein	143	4–4	6
	bFGF mRNA	94	15–13	5

Incubation of rat skeletal muscle cells with donors of prostanoids (iloprost, TXB, PF or PE) induced no changes in VEGF or bFGF mRNA levels or in release of VEGF protein. Indomethacin incubation induced no change in bFGF mRNA content. Values are geometric means. All interventions are non-significant. These data are not presented in figures.

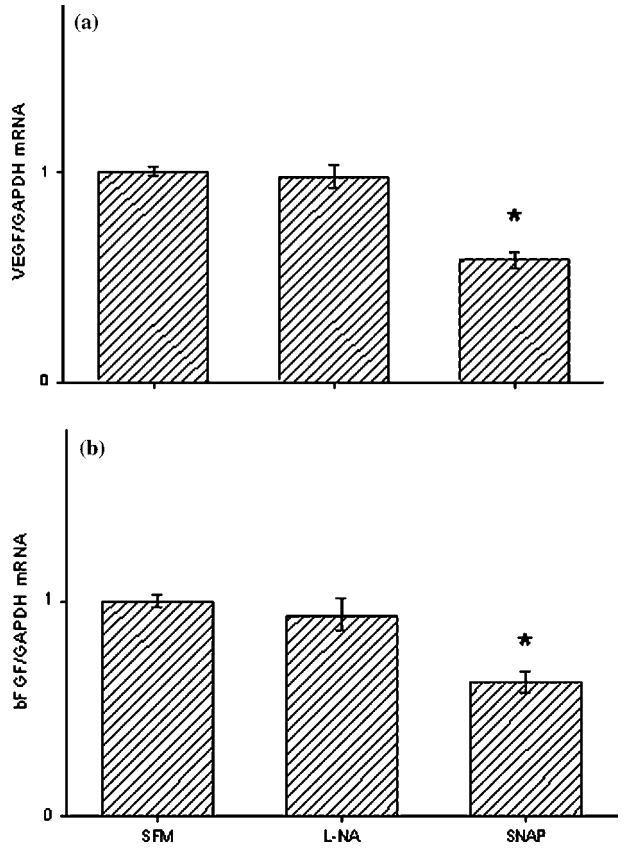

Figure 3. Effect of L-NA or SNAP on VEGF and bFGF mRNA in rat skeletal muscle cells. Primary muscle cells were incubated for 24 h with serum free medium (SFM; $n = 18$), 1 mM NG-nitro-L-arginine (L-NA; $n = 18$) or 0.5 μM *S*-nitroso-*N*-acetyl-D, L-penicillamine (SNAP; $n = 9$) and cells analysed for (a) VEGF mRNA and (b) bFGF mRNA. Values are relative to SFM. Results are obtained from three (SNAP) or five (SFM and L-NA) independent experiments. *$P < 0.05$ vs SFM.

Table 3. Effect of L-NA and SNAP on VEGF and bFGF protein in primary rat skeletal muscle cells.

Treatment	Measurement	Relative to control (=100)	SE+ and SE−	*n*
L-NA	VEGF protein	90	10–9	14
SNAP	VEGF protein	122	18–16	13

Incubation of rat skeletal muscle cells with the NO inhibitor, L-NA or the NO donor, SNAP, induced no changes in the release of VEGF protein. Values are geometric means. All interventions are non-significant. These data are not presented in figures.

Table 4. Effect of AOPCP and adenosine on VEGF and bFGF mRNA in rat skeletal muscle cells.

Treatment	Measurement	Relative to control (=100)	SE+ and SE−	*n*
AOPCP	VEGF mRNA	101	14–13	15
	bFGF mRNA	78	7–6	16
Adenosine	VEGF mRNA	81	5–5	2
	bFGF mRNA	77	2–2	3

Incubation of rat skeletal muscle cells with an inhibitor of adenosine formation, AOPCP or with adenosine, induced no changes in VEGF or bFGF mRNA content. Values are geometric means. All interventions are non-significant. These data are not presented in figures.

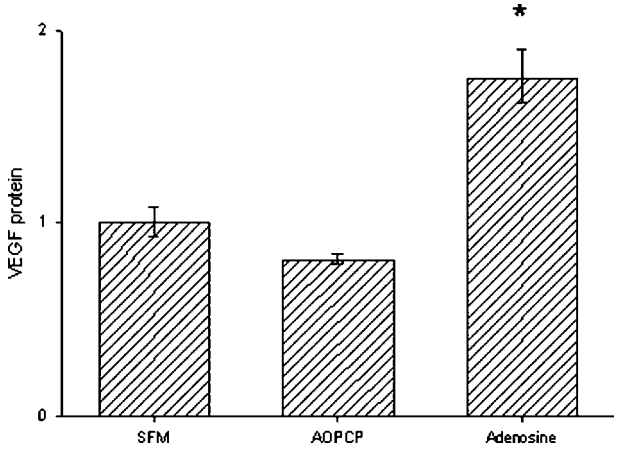

Figure 4. Effect of AOPCP and adenosine on extracellular VEGF protein in rat skeletal muscle cells. Primary muscle cells were incubated for 24 h with serum free medium (SFM; $n = 10$), 50 μM AOPCP (an inhibitor of ecto 5′-nucleotidase; $n = 10$) or 0.2 mM adenosine ($n = 4$). Medium was analysed for VEGF protein content. Values are relative to SFM. Results are obtained from two independent experiments. *$P < 0.05$ vs SFM.

mRNA levels. Addition of adenosine lead to a 75% increase in extracellular VEGF ($P < 0.05$) whereas AOPCP did not alter the VEGF protein levels (Figure 4).

bFGF mRNA. bFGF mRNA levels were unaffected by incubation with either AOPCP or with adenosine compared to control (Table 4).

Endothelial cell proliferation

Effect of medium from electro-stimulated rat skeletal muscle cells on endothelial cell proliferation

Incubation of endothelial cells with medium from electro-stimulated muscle cells did not induce a greater cell proliferation relative to medium from non-stimulated muscle cells at any time-point (Table 5).

Effect of medium from indomethacin and prostanoid treated skeletal muscle cells on endothelial cell proliferation

No differences ($P > 0.05$) in endothelial cell proliferation were observed using medium from muscle cells that had been treated with indomethacin or with prostanoid donors in combination with indomethacin (Table 6).

Effect of medium from L-NA and SNAP-treated rat muscle cells on endothelial cell proliferation

Endothelial cell proliferation was not affected ($P > 0.05$) by medium from muscle cells treated with L-NA, but was decreased by 45% ($P < 0.05$) when using medium from muscle cells treated with SNAP (Figure 5).

Effect of medium from AOPCP and adenosine-treated rat muscle cells on endothelial cell proliferation

Medium from muscle cells incubated with AOPCP had no effect ($P > 0.05$) on endothelial cell proliferation whereas medium from muscle cells that had been incubated with adenosine enhanced the proliferative effect by 23% ($P < 0.05$) compared to medium from control muscle (Figure 5).

Table 5. Effect of extracellular medium from non-stimulated and electro-stimulated rat skeletal muscle cells on endothelial cell proliferation.

Treatment	Measurement	Relative to control (=100)	SE+ and SE−	*n*
Non-stim 10 h	EC proliferation	92	8–7	2
Non-stim 24 h	EC proliferation	104	10–9	12
Stim 0 h	EC proliferation	99	10–9	12
Stim 10 h	EC proliferation	93	3–3	2
Stim 24 h	EC proliferation	80	8–7	12

Medium obtained at 0 or 24 h after electro-stimulation or non-stimulation of rat skeletal muscle cells induced no changes in endothelial cell proliferation. Values are geometric means. All interventions are non-significant. These data are not presented in figures.

Table 6. Effect of extracellular medium from skeletal muscle cells treated with cyclooxygenase inhibitor and prostanoids on endothelial cell proliferation.

Treatment	Measurement	Relative to control (=100)	SE+ and SE−	*n*
Indomethacin	EC proliferation	67	9–8	14
Indo + iloprost	EC proliferation	81	8–7	14
Indo + TXB	EC proliferation	82	12–10	14
Indo + PF	EC proliferation	76	5–5	6
Indo + PE	EC proliferation	83	3–3	5

Medium obtained from rat skeletal muscle cells that have been incubated with indomethacin alone or in combination with prostanoids donors (iloprost, TXB, PF or PE) showed no changes in endothelial cell proliferation. Values are geometric means. All interventions are non-significant. These data are not presented in figures.

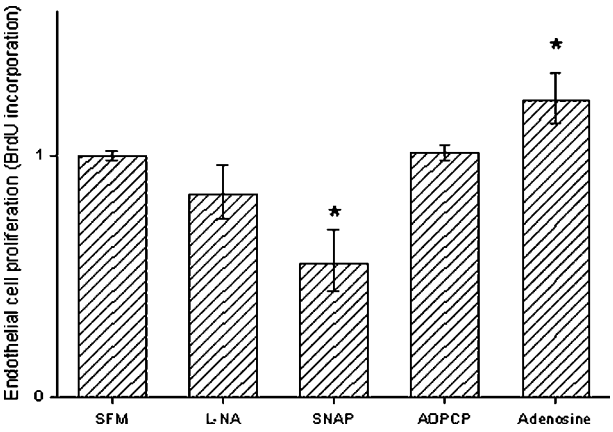

Figure 5. Effect of treatment of rat skeletal muscle cells with L-NA, SNAP, AOPCP or adenosine on the endothelial cell proliferative effect of muscle extracellular medium. Endothelial cell proliferation was measured after addition of medium from muscle cells incubated with serum free medium (SFM; $n = 18$), 1 mM NG-nitro-L-arginine (L-NA; $n = 14$), 0.5 μM *S*-nitroso-*N*-acetyl-D, L-penicillamine (SNAP; $n = 13$), 50 μM AOPCP (an inhibitor of ecto 5′-nucleotidase; $n = 18$) or 0.2 mM adenosine ($n = 4$). Values are relative to SFM. Results are obtained from two (AOPCP and adenosine) or four (SFM, L-NA and SNAP) independent experiments. *$P < 0.05$ vs SFM.

Regulation of VEGF and bFGF in human skeletal muscle cells

Effect of electro-stimulation on VEGF and bFGF in human skeletal muscle cells

VEGF mRNA and protein. A significant interaction ($P < 0.05$) between time and stimulation was found and VEGF mRNA content in electro-stimulated human muscle cells was higher at 24 h than at 0 hour after the stimulation ($P < 0.05$). At 24 h after electro-stimulation VEGF mRNA levels in the muscle cells were 24% higher ($P < 0.05$) than in non-stimulated cells (Figure 6a).

Electro-stimulation of human muscle cells did not induce a greater concentration of extracellular VEGF release than that of non-stimulated control cells at any time point ($P > 0.05$). Incubation for 24 h elicited a 4-fold increase in the VEGF release ($P < 0.05$) (Figure 6b).

bFGF mRNA. Electro-stimulation of human muscle cells did not alter ($P > 0.05$) the level of bFGF mRNA content. bFGF mRNA content was 40% higher at the 24 h time point than at 0 h both for non-stimulated and electrostimulated cells (Figure 6c).

Effect of medium from electro-stimulated human muscle cells on endothelial cell proliferation

Medium from stimulated human muscle cells induced an increase ($P < 0.05$) in endothelial cell proliferation compared to medium from control cells. The proliferation was not dependent on incubation time of medium with muscle cells (Figure 6d).

Effect of actinomycin-D on mRNA levels

The half-life for VEGF mRNA after actinomycin-D treatment was 3.92, 3.25 and 3.8 h for control, electro-

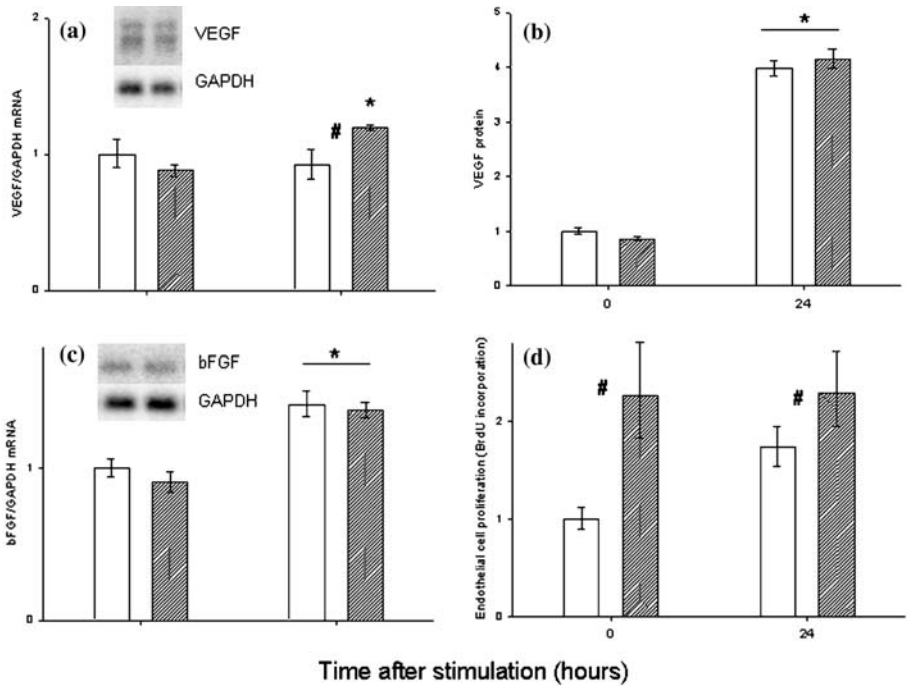

Figure 6. Effect of time and electro-stimulation of human skeletal muscle cells on VEGF and bFGF expression, VEGF extracellular protein and endothelial cell proliferation. Non-stimulated (white bars) or electrically stimulated (grey bars) primary human skeletal muscle cells were after 0 or 24 h after treatment analysed for (a) VEGF mRNA (b) VEGF protein in medium, (c) bFGF mRNA or (d) the effect of medium on endothelial cell proliferation. Values are relative to non-stimulated cells at 0 h. Insert: representative bands of VEGF and bFGF mRNA and GAPDH obtained by northern blot. $n = 3$ in 0 hour and $n = 6$ in 24 h condition. Results are obtained from one experiment. $^{*}P < 0.05$ vs 0 h, $^{\#}P < 0.05$ non-stimulated vs stimulated.

stimulated and indomethacin-treated muscle cells, respectively (Figure 7a).

The half-life of bFGF mRNA after actinomycin-D treatment was 4.75, 4.45 and 5.4 h for control, electro-stimulated and indomethacin-treated muscle cells, respectively (Figure 7b).

Discussion

The results of the present study show that electro-stimulation of skeletal muscle cells induces an increase in VEGF and bFGF mRNA content whereas exogenous NO reduces the VEGF and bFGF mRNA level. Cyclooxygenase inhibition of skeletal muscle cells leads to an increased VEGF mRNA level and an enhanced extracellular VEGF protein concentration, effects that are absent after simultaneous addition of $PF_{2\alpha}$, PE_2 and TXB_2. Electro-stimulation of human, but not rat, skeletal muscle cells leads to a higher proliferative effect of the extracellular medium on endothelial cells than medium from non-stimulated muscle cells. The endothelial cell proliferative effect of rat skeletal muscle cell extracellular medium was higher after treatment of the muscle cells with adenosine and lower after treatment with an NO donor. Thus, contractile activity, prostanoids and NO appear to be involved in the regulation of angiogenic compounds, including VEGF, produced by skeletal muscle.

Muscle contraction

VEGF and bFGF mRNA have, by *in situ* hybridisation and histochemical techniques been shown to be present in the cytosol and sarcolemma of human skeletal muscle tissue [33, 34]. Studies on cells in culture have, similarly shown VEGF mRNA to be present in the C2C12 muscle cell line, whereas bFGF mRNA has in one previous study been shown to be present [35] and in one study been shown to be absent [36] in rat primary skeletal muscle cell cultures. The present study shows presence of VEGF and bFGF in both human and rat primary skeletal muscle cell cultures and suggests that both growth factors indeed are expressed in skeletal muscle cells.

Studies performed *in vivo* have shown that one bout of exercise [37–40] induces an increase in VEGF mRNA in homogenates of both rat and human skeletal muscle cells. The results of the present study of an increase in VEGF mRNA in both rat and human primary skeletal muscle cells suggest that the observed increase in VEGF mRNA in homogenates, at least in part, is due to increases occurring in the skeletal muscle cells. The increase in VEGF mRNA levels in the muscle cells observed in the present study was of a somewhat slower time-course than observed *in vivo*. VEGF mRNA content in muscle tissue *in vivo* has been shown to increase immediately or shortly after an exercise bout [41–44] and return to basal levels 24 h after the stimulation [45]. In the present study, as well as in a previous study examining the murine muscle cell line C2C12 [46], VEGF mRNA in the muscle cell cultures was significantly increased first at 22–24 h after electro-stimulation.

The VEGF protein concentration in the extracellular medium increased from 0 to 24 h in the non-stimulated muscle cell cultures suggesting that there is a basal release

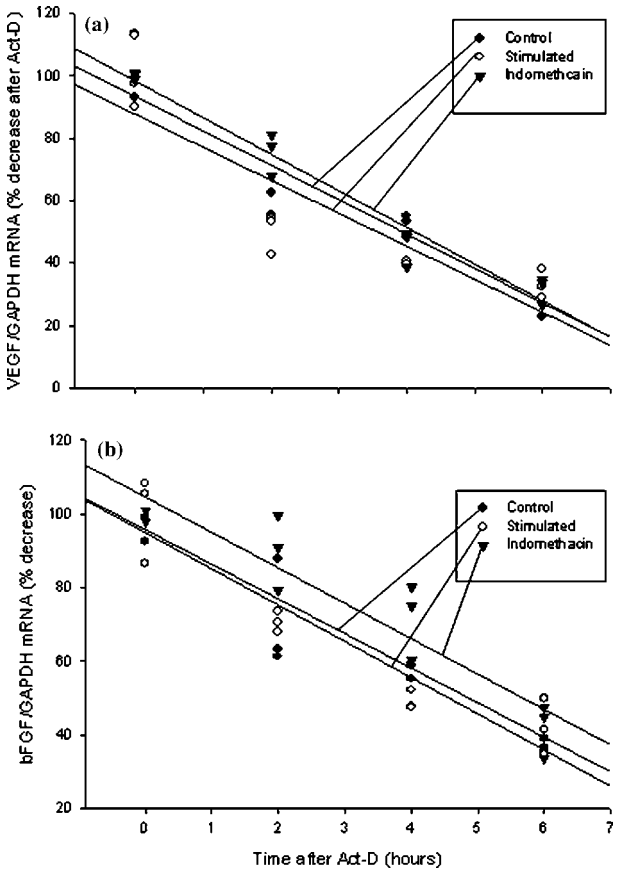

Figure 7. Effect of actinomycin-D on mRNA levels in skeletal muscle. Primary skeletal muscle cells were incubated for 0, 2, 4 or 6 h with 5.3 μM actinomycin-D and cells were analysed for (a) VEGF mRNA and (b) bFGF mRNA. The average of the 0 h value was set to 100 and individual values were plotted relative to this value. $n = 3$. Results are obtained from one experiment.

of VEGF from muscle cells. This is probably the result of a basal release of VEGF from the muscle cells, and it may be hypothesised that this basal release from muscle cells is involved in the maintenance of capillarisation in the muscle tissue. A direct relationship between the extracellular VEGF protein concentration and the VEGF mRNA level was not evident as despite the 33% higher level of VEGF mRNA in the electro-stimulated cells, the release of VEGF protein was similar to that released from the non-stimulated control cells. It cannot be excluded, however, that the assessment period of VEGF protein within 24 h of electro-stimulation was insufficient considering that the increase in VEGF mRNA was significant first at 24 h after electro-stimulation.

In the present study, electro-stimulation of muscle cells lead to an increase in bFGF mRNA levels in the rat muscle cells but not in the human muscle cells. This observation is in congruence with *in vivo* observations of an exercise-induced increase in bFGF mRNA content in rat [47] but not in human [48, 49]. The increase in bFGF mRNA was observed immediately after electro-stimulation of the rat muscle cells which is a similar time course to that previously reported for rat skeletal muscle showing an increase in 0 h after 1 h of treadmill running [50]. This observation suggests that, at least in rat, skeletal muscle cells are not only a potential source of VEGF but also of bFGF.

Prostanoids

We investigated the regulatory role of prostanoids for VEGF and bFGF in skeletal muscle. A 170% increase in VEGF mRNA in muscle cells was observed after inhibition of COX by indomethacin incubation. The increased VEGF mRNA levels in the indomethacin-treated muscle cells was paralleled by an enhanced release of VEGF protein from the muscle cells as evidenced by an approximate 100% increase in extracellular VEGF protein concentration compared to control cells. To our knowledge, the effect of COX inhibition on VEGF and bFGF expression in muscle cell cultures has not previously been investigated, however an *in vivo* study showed a decrease in VEGF and bFGF mRNA in whole homogenates of rat skeletal muscle after arterial infusion of PGI_2 [51]. Combined, these studies suggest that prostanoids restrict the level of VEGF mRNA in skeletal muscle cells. The importance of prostanoids in the regulation of VEGF expression was investigated by a combined addition of indomethacin and donors of prostanoids (iloprost, TXB, $PF_{2\alpha}$, PE_2), all of which have previously been shown to have an effect on angiogenesis and/or growth factor expression [52–59]. Addition of indomethacin in combination with either TXB, $PF_{2\alpha}$ or PE_2, but not iloprost, to muscle cells did not induce an increase in VEGF mRNA or VEGF extracellular concentration as observed with indomethacin alone. These results indicate that prostaglandins and TXA_2 all have an inhibitory effect on VEGF mRNA expression and VEGF protein levels in muscle cells whereas prostacyclin does not appear to be involved. The addition of any of the prostanoids without indomethacin did not affect basal VEGF mRNA expression or VEGF release in the muscle cells. Based on these observations it could thus be speculated that prostanoids are involved in the regulation of a stimuli-induced increase in VEGF mRNA levels but not in the maintenance of basal VEGF mRNA levels. Alternatively, the results may imply that the basal production of prostanoids is sufficient to inhibit VEGF mRNA and that addition of extra prostanoids does not cause further inhibition.

The specificity of indomethacin in the present study was verified by measurements of PGI_2 release from the skeletal muscle cells where incubation of skeletal muscle cells with indomethacin was found to reduce the contraction-induced release by 67%. The specificity of indomethacin was furthermore verified by the reversal of the indomethacin-induced increase in VEGF mRNA expression and VEGF release, by addition of TXB, PE_2 or $PF_{2\alpha}$.

Nitric oxide

NO has been reported to be involved in the regulation of VEGF and bFGF mRNA [60, 61] and studies have shown that NO is formed in cultured rat skeletal muscle cells [62]. However, when muscle cells were incubated with the NOS inhibitor, L-NA, in the present study, no effect on either VEGF or bFGF mRNA content or VEGF release was observed. These results are in accordance with findings in rat muscle *in vivo* where NOS inhibition was found not to

affect either basal VEGF or bFGF mRNA expression [63]. The efficacy of L-NA in muscle cell-cultures has previously been verified in our laboratory where NO formation in non-stimulated as well as electro-stimulated rat primary skeletal muscle cells was shown to be eliminated by addition of L-NA at a concentration lower than that used in the present study (0.3 vs 1 mM) [64].

Addition of exogenous NO *via* the donor SNAP did, however, decrease the content of both VEGF and bFGF mRNA. This is somewhat in contrast with findings in an *in vivo* study from Benoit et al. [65] showing an increase in VEGF mRNA and no effect on bFGF mRNA, in rat muscle homogenate after arterial infusion of the NO donor nitroprusside. The discrepancy suggests that either NO acts in concert with other factors *in vivo* or the effect of NO *in vivo* is mediated *via* another cell-type, e.g. endothelial cells. Nevertheless, our results show that the isolated effect of exogenous NO on skeletal muscle cells is in lowering VEGF mRNA. It is unlikely that the decreased growth factor mRNA level and growth inhibitory effect of NO (SNAP) were due to cytotoxicity since the amount of total RNA was not affected by SNAP incubation (results not shown). Furthermore a study by RayChaudhury et al. [66] showed that the effect of SNAP (200 μM) on bovine arterial endothelial cells was reversible upon removal and furthermore, SNAP did not reduce the number of cells.

Adenosine

In the present study, incubation of muscle cells with adenosine elicited an increased release of VEGF. On the other hand, no effect of inhibition of adenosine formation was observed on VEGF or bFGF mRNA expression or VEGF release in muscle cell cultures. The observation suggests that the release of VEGF protein from the muscle cells may be regulated by adenosine. The role of adenosine for VEGF and bFGF expression has not previously been examined in skeletal muscle cell cultures, however, an *in vivo* study showed that infusion of adenosine in rats had no effect on the expression of these growth factors [67]. In cultured primary rat skeletal muscle cells, it has been observed that the effect of adenosine on adenylate cyclase activation is mainly mediated *via* the A_{2B} receptor [68]. Thus, the present observed effects of adenosine on skeletal muscle cells suggest that the effect of adenosine on VEGF release may be mediated *via* activation of the A_{2B} receptor whereas this pathway apparently does not regulate VEGF mRNA expression.

Endothelial cell proliferation

Electro-stimulation enhanced the endothelial cell proliferative effect of the extracellular medium from the human muscle cell cultures. This observation is in congruence with our previous findings in a microdialysis study on human subjects, showing a greater proliferative effect for human skeletal muscle interstitial fluid after muscle contraction [69, 70]. The results suggest that at least part of the proliferative effect of muscle interstitial fluid can be explained by release of angiogenic compounds from the skeletal muscle cells.

In contrast to the effect of electro-stimulation on human muscle cells, contractile activity of rat skeletal muscle cells did not enhance the proliferative effect of the extracellular medium. This discrepancy between human and rat cells could potentially be explained by the fact that the extracellular medium from the human muscle cells contains a compound that is specific for human endothelial cells and that does not exist or does not function in medium from rat muscle cells. It is, however, unlikely that such a compound would be VEGF, since VEGF from mice, rats and human cells have all been shown to induce increase, in proliferation or COX expression in both HUVECs or bovine endothelial cells [71–73].

In general, it may be questioned to what extent VEGF contributes to the contraction-induced increase in proliferative effect of the human muscle extracellular fluid. The elevation in the level of VEGF mRNA by electro-stimulation was similar in the rat and human skeletal muscle cells yet only the human muscle cell extracellular medium showed an increased proliferative effect. Moreover, despite a 2-fold increase in VEGF protein in the extracellular medium of the indomethacin-treated rat muscle cells, the effect on endothelial cell proliferation was similar to that of control medium. Medium from muscle cells incubated with SNAP decreased endothelial cell proliferation without affecting the extracellular VEGF protein levels in comparison to medium from control muscle cells. Thus, there appears to be no direct relationship between the level of VEGF in the muscle cell cultures and the proliferative effect of the muscle extracellular fluid.

Control experiments were conducted to assess whether the effect of SNAP on lowering endothelial cell proliferation was an effect on the endothelium or an effect mediated via the muscle cells. Medium from endothelial cells incubated with SNAP showed no changes in endothelial cell proliferation (L. Jensen, data not shown), suggesting that the observed decrease in proliferation, with medium from SNAP treated muscle cells, was not due to a direct effect of SNAP. Thus, it appears that NO regulates the release of an angiogenic compound from the skeletal muscle cells, a compound that is unlikely to be VEGF.

As bFGF has been shown to increase angiogenesis [74] and endothelial cell proliferation [75], it can be speculated that the decreased proliferative effect of medium from the SNAP-treated muscle cells was associated with the reduced amount of bFGF mRNA and a potential lowering of bFGF protein in these cells. However, although this possibility cannot be excluded, it is not very likely considering the low levels of bFGF in the medium of the control muscle cells in the present study. The observed effects of NO on endothelial cell proliferation is in agreement with several studies [76–79] and suggest that the increased formation of NO by muscle cells that occurs in response to exercise [80] could restrict proliferation of endothelial cells.

Medium from muscle cells treated with adenosine elicited an increase in endothelial cell proliferation compared to medium from control cells, whereas medium from AOPCP-

treated muscle cells elicited no changes in endothelial cell proliferation. The present results suggest that the proliferative effect of adenosine might be mediated through release of proliferative compounds from muscle cells. In the light of adenosine enhancing the basal release of VEGF from muscle cells, one such proliferative compound may be VEGF.

Stability of mRNA

The level of expression of VEGF and bFGF mRNA is determined by the transcription rate of genes and/or the stability of the mRNA. Therefore, to evaluate the stability of VEGF and bFGF mRNA, actinomycin-D, which blocks transcription, was added to cell cultures. There were no differences in half-lives calculated in control, electrically stimulated or indomethacin-treated cells of either VEGF or bFGF mRNA. Thus, the electrical stimulation or indomethacin treatment did not affect the stability of VEGF and bFGF mRNA, indicating that changes in VEGF and bFGF mRNA were at the transcriptional level.

Conclusion

In summary, this study provides evidence that a major source of the contraction-induced increase in VEGF expression might be the muscle cells. Contraction of skeletal muscle cells, as well as prostanoids, NO and adenosine regulate the level of VEGF and bFGF mRNA in skeletal muscle cells with TXA_2, $PF_{2\alpha}$, PE_2 and NO restricting VEGF expression, and adenosine enhancing VEGF release. In spite of a similar effect on growth factor expression, NO decreases the proliferative effect while prostanoids increase the proliferative effect of muscle cell extracellular medium, suggesting that muscle cells also produce proliferative compounds other than VEGF and bFGF.

References

1. Andersen P, Henriksson J. Capillary supply of the quadriceps femoris muscle of man: Adaptive response to exercise. J Physiol 1977; 270(3): 677–90.
2. Goto F, Goto K, Weindel K, Folkman J. Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels [see comments]. Lab Invest 1993; 69(5): 508–17.
3. Leung DW, Cachianes G, Kuang WJ et al. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989; 246(4935): 1306–09.
4. Breen EC, Johnson EC, Wagner H et al. Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. J Appl Physiol 1996; 81(1): 355–61.
5. Hoffner L, Nielsen JJ, Langberg H, Hellsten Y. Exercise but not prostanoids enhance levels of vascular endothelial growth factor and other proliferative agents in human skeletal muscle interstitium. J Physiol 2003; 550(Pt 1): 217–25.
6. Peters KG, Werner S, Chen G, Williams LT. Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. Development 1992; 114(1): 233–43.
7. Ferrara N, Houck K, Jakeman L, Leung DW. Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocr Rev 1992; 13(1): 18–32.
8. Kanno S, Oda N, Abe M et al. Establishment of a simple and practical procedure applicable to therapeutic angiogenesis. Circulation 1999; 99(20): 2682–87.
9. Frandsen U, Bangsbo J, Langberg H et al. Inhibition of nitric oxide synthesis by systemic N(G)-monomethyl-L-arginine administration in humans: Effects on interstitial adenosine, prostacyclin and potassium concentrations in resting and contracting skeletal muscle. J Vasc Res 2000; 37(4): 297–302.
10. Karamouzis M, Karamouzis I, Vamvakoudis E et al. The response of muscle interstitial prostaglandin E(2)(PGE(2)), prostacyclin I(2)(PGI(2)) and thromboxane A(2)(TXA(2)) levels during incremental dynamic exercise in humans determined by *in vivo* microdialysis. Prostaglandins Leukot Essent Fatty Acids 2001; 64(4–5): 259–63.
11. Hoffner L, Nielsen JJ, Langberg H, Hellsten Y. Exercise but not prostanoids enhance levels of vascular endothelial growth factor and other proliferative agents in human skeletal muscle interstitium. J Physiol 2003; 550(Pt 1): 217–25.
12. Benoit H, Jordan M, Wagner H, Wagner PD. Effect of NO, vasodilator prostaglandins, and adenosine on skeletal muscle angiogenic growth factor gene expression. J Appl Physiol 1999; 86(5): 1513–18.
13. Jones MK, Wang H, Peskar BM et al. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: Insight into mechanisms and implications for cancer growth and ulcer healing. Nat Med 1999; 5(12): 1418–23.
14. Murphy JF, Steele C, Belton O, Fitzgerald DJ. Induction of cyclooxygenase-1 and -2 modulates angiogenic responses to engagement of alphavbeta3. Br J Haematol 2003; 121(1): 157–64.
15. BenEzra D. Neovasculogenic ability of prostaglandins, growth factors, and synthetic chemoattractants. Am J Ophthalmol 1978; 86(4): 455–61.
16. Benoit H, Jordan M, Wagner H, Wagner PD. Effect of NO, vasodilator prostaglandins, and adenosine on skeletal muscle angiogenic growth factor gene expression. J Appl Physiol 1999; 86(5): 1513–18.
17. Hoper MM, Voelkel NF, Bates TO et al. Prostaglandins induce vascular endothelial growth factor in a human monocytic cell line and rat lungs via cAMP. Am J Respir Cell Mol Biol 1997; 17(6): 748–56.
18. Benoit H, Jordan M, Wagner H, Wagner PD. Effect of NO, vasodilator prostaglandins, and adenosine on skeletal muscle angiogenic growth factor gene expression. J Appl Physiol 1999; 86(5): 1513–18.
19. Harada S, Nagy JA, Sullivan KA et al. Induction of vascular endothelial growth factor expression by prostaglandin E2 and E1 in osteoblasts. J Clin Invest 1994; 93(6): 2490–96.
20. Pearce SC, Hudlicka O, MD. Effect of indomethacin on capillary growth and microvasculature in chronically stimulated rat skeletal muscles. J Physiol 2000; 526 (Pt 2): 435–43.
21. Benoit H, Jordan M, Wagner H, Wagner PD. Effect of NO, vasodilator prostaglandins, and adenosine on skeletal muscle angiogenic growth factor gene expression. J Appl Physiol 1999; 86(5): 1513–18.
22. Gavin TP, Spector DA, Wagner H et al. Nitric oxide synthase inhibition attenuates the skeletal muscle VEGF mRNA response to exercise. J Appl Physiol 2000; 88(4): 1192–98.
23. RayChaudhury A, Frischer H, Malik AB. Inhibition of endothelial cell proliferation and bFGF-induced phenotypic modulation by nitric oxide. J Cell Biochem 1996; 63(2): 125–34.
24. Lee PC, Kibbe MR, Schuchert MJ et al. Nitric oxide induces angiogenesis and upregulates alpha(v)beta(3) integrin expression on endothelial cells. Microvasc Res 2000; 60(3): 269–80.
25. Hashimoto E, Kage K, Ogita T et al. Adenosine as an endogenous mediator of hypoxia for induction of vascular endothelial growth factor mRNA in U-937 cells. Biochem Biophys Res Commun 1994; 204(1): 318–24.
26. Gu JW, Brady AL, Anand V et al. Adenosine upregulates VEGF expression in cultured myocardial vascular smooth muscle cells. Am J Physiol 1999; 277(2 Pt 2): H595–602.
27. Bergström J. Muscle electrolytes in man. Determined by neuron activation analysis on needle biopsy specimen. A study on normal subjects, kidney patients and patients with chronic diarrhoea. Scand J Clin Lab Invest 1962; 14: 87–93.

28. Hellsten Y, Frandsen U. Adenosine formation in contracting primary rat skeletal muscle cells and endothelial cells in culture. J Physiol 1997; 504(Pt 3): 695–704.
29. Jonsdottir IH, Schjerling P, Ostrowski K et al. Muscle contractions induce interleukin-6 mRNA production in rat skeletal muscles. J Physiol 2000; 528(Pt 1): 157–63.
30. Higginson J, Wackerhage H, Woods N et al. Blockades of mitogen-activated protein kinase and calcineurin both change fibre-type markers in skeletal muscle culture. Pflugers Arch 2002; 445(3): 437–43.
31. Tischer E, Mitchell R, Hartman T et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 1991; 266(18): 11947–54.
32. Cheung CY, Singh M, Ebaugh MJ, Brace RA. Vascular endothelial growth factor gene expression in ovine placenta and fetal membranes. Am J Obstet Gynecol 1995; 173(3 Pt 1): 753–59.
33. Gustafsson T, Bodin K, Sylven C et al. Increased expression of VEGF following exercise training in patients with heart failure. Eur J Clin Invest 2001; 31(4): 362–66.
34. Jensen L, Bangsbo J, Hellsten Y. Effect of high intensity training on capillarization and presence of angiogenic factors in human skeletal muscle. J Physiol 2004; 557(Pt 2): 571–82.
35. Kastner S, Elias MC, Rivera AJ, Yablonka-Reuveni Z. Gene expression patterns of the fibroblast growth factors and their receptors during myogenesis of rat satellite cells. J Histochem Cytochem 2000; 48(8): 1079–96.
36. Alterio J, Courtois Y, Robelin J et al. Acidic and basic fibroblast growth factor mRNAs are expressed by skeletal muscle satellite cells. Biochem Biophys Res Commun 1990; 166(3): 1205–12.
37. Breen EC, Johnson EC, Wagner H et al. Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. J Appl Physiol 1996; 81(1): 355–61.
38. Gustafsson T, Puntschart A, Kaijser L et al. Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. Am J Physiol 1999; 276(2 Pt 2): H679–85.
39. Richardson RS, Wagner H, Mudaliar SR et al. Human VEGF gene expression in skeletal muscle: Effect of acute normoxic and hypoxic exercise. Am J Physiol 1999; 277(6 Pt 2): H2247–52.
40. Gavin TP, Wagner PD. Effect of short-term exercise training on angiogenic growth factor gene responses in rats. J Appl Physiol 2001; 90(4): 1219–26.
41. Breen EC, Johnson EC, Wagner H et al. Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. J Appl Physiol 1996; 81(1): 355–61.
42. Gustafsson T, Puntschart A, Kaijser et al. Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. Am J Physiol 1999; 276(2 Pt 2): H679-85.
43. Richardson RS, Wagner H, Mudaliar SR et al. Human VEGF gene expression in skeletal muscle: Effect of acute normoxic and hypoxic exercise. Am J Physiol 1999; 277(6 Pt 2): H2247–52.
44. Gavin TP, Wagner PD. Effect of short-term exercise training on angiogenic growth factor gene responses in rats. J Appl Physiol 2001; 90(4): 1219–26.
45. Jensen L, Pilegaard H, Neufer PD, Hellsten Y. Effect of acute exercise and Exercise training on VEGF splice variants in human Skeletal Muscle. Am J Physiol Regul Integr Comp Physiol 2004.
46. Kanno S, Oda N, Abe M et al. Establishment of a simple and practical procedure applicable to therapeutic angiogenesis. Circulation 1999; 99(20): 2682–87.
47. Breen EC, Johnson EC, Wagner H et al. Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. J Appl Physiol 1996; 81(1): 355–61.
48. Gustafsson T, Puntschart A, Kaijser L et al. Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. Am J Physiol 1999; 276(2 Pt 2): H679–85.
49. Richardson RS, Wagner H, Mudaliar SR et al. Human VEGF gene expression in skeletal muscle: Effect of acute normoxic and hypoxic exercise. Am J Physiol 1999; 277(6 Pt 2): H2247–52.
50. Breen EC, Johnson EC, Wagner H et al. Angiogenic growth factor mRNA responses in muscle to a single bout of exercise. J Appl Physiol 1996; 81(1): 355–61.
51. Benoit H, Jordan M, Wagner H, Wagner PD. Effect of NO, vasodilator prostaglandins, and adenosine on skeletal muscle angiogenic growth factor gene expression. J Appl Physiol 1999; 86(5): 1513–18.
52. Hoper MM, Voelkel NF, Bates TO et al. Prostaglandins induce vascular endothelial growth factor in a human monocytic cell line and rat lungs via cAMP. Am J Respir Cell Mol Biol 1997; 17(6): 748–56.
53. Jones MK, Wang H, Peskar BM et al. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: Insight into mechanisms and implications for cancer growth and ulcer healing. Nat Med 1999; 5(12): 1418–23.
54. Knox AJ, Corbett L, Stocks J et al. Human airway smooth muscle cells secrete vascular endothelial growth factor: Up-regulation by bradykinin via a protein kinase C and prostanoid-dependent mechanism. FASEB J 2001; 15(13): 2480–8.
55. Sakai Y, Fujita K, Sakai H, Mizuno K. Prostaglandin E2 regulates the expression of basic fibroblast growth factor messenger RNA in normal human fibroblasts. Kobe J Med Sci 2001; 47(1): 35–45.
56. Pai R, Szabo IL, Soreghan BA et al. PGE(2) stimulates VEGF expression in endothelial cells via ERK2/JNK1 signaling pathways. Biochem Biophys Res Commun 2001; 286(5): 923–8.
57. Murphy JF, Steele C, Belton O, Fitzgerald DJ. Induction of cyclooxygenase-1 and -2 modulates angiogenic responses to engagement of alphavbeta3. Br J Haematol 2003; 121(1): 157–64.
58. BenEzra D. Neovasculogenic ability of prostaglandins, growth factors, and synthetic chemoattractants. Am J Ophthalmol 1978; 86(4): 455–61.
59. Nie D. Lamberti M, Zacharek A et al. Thromboxane A(2) regulation of endothelial cell migration, angiogenesis, and tumor metastasis. Biochem Biophys Res Commun 2000; 267(1): 245–51.
60. Fukuo K, Inoue T, Morimoto S et al. Nitric oxide mediates cytotoxicity and basic fibroblast growth factor release in cultured vascular smooth muscle cells. A possible mechanism of neovascularization in atherosclerotic plaques. J Clin Invest 1995; 95(2): 669–76.
61. Dulak J, Jozkowicz A, Dembinska-Kiec A et al. Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 2000; 20(3): 659–66.
62. Silveira LR, Pereira-Da-Silva L et al. Formation of hydrogen peroxide and nitric oxide in rat skeletal muscle cells during contractions. Free Radic Biol Med 2003; 35(5): 455–64.
63. Gavin TP, Spector DA, Wagner H et al. Nitric oxide synthase inhibition attenuates the skeletal muscle VEGF mRNA response to exercise. J Appl Physiol 2000; 88(4): 1192–98.
64. Silveira LR, Pereira-Da-Silva L, Juel C, Hellsten Y. Formation of hydrogen peroxide and nitric oxide in rat skeletal muscle cells during contractions. Free Radic Biol Med 2003; 35(5): 455–64.
65. Benoit H, Jordan M, Wagner H, Wagner PD. Effect of NO, vasodilator prostaglandins, and adenosine on skeletal muscle angiogenic growth factor gene expression. J Appl Physiol 1999; 86(5): 1513–8.
66. RayChaudhury A, Frischer H, Malik AB. Inhibition of endothelial cell proliferation and bFGF-induced phenotypic modulation by nitric oxide. J Cell Biochem 1996; 63(2): 125–34.
67. Benoit H, Jordan M, Wagner H, Wagner PD. Effect of NO, vasodilator prostaglandins, and adenosine on skeletal muscle angiogenic growth factor gene expression. J Appl Physiol 1999; 86(5): 1513–8.
68. Lynge J, Schulte G, Nordsborg N et al. Adenosine A 2B receptors modulate cAMP levels and induce CREB but not ERK1/2 and p38 phosphorylation in rat skeletal muscle cells. Biochem Biophys Res Commun 2003; 307(1): 180–7.
69. Hoffner L, Nielsen JJ, Langberg H, Hellsten Y. Exercise but not prostanoids enhance levels of vascular endothelial growth factor and other proliferative agents in human skeletal muscle interstitium. J Physiol 2003; 550(Pt 1): 217–25.
70. Jensen L, Bangsbo J, Hellsten Y. Effect of high intensity training on capillarization and presence of angiogenic factors in human skeletal muscle. J Physiol 2004; 557(Pt 2): 571–82.
71. Kanno S, Oda N, Abe M et al. Establishment of a simple and practical procedure applicable to therapeutic angiogenesis. Circulation 1999; 99(20): 2682–7.

72. Kosmidou I, Xagorari A, Roussos C, Papapetropoulos A. Reactive oxygen species stimulate VEGF production from C(2)C(12) skeletal myotubes through a PI3K/Akt pathway. Am J Physiol Lung Cell Mol Physiol 2001; 280(4): L585–92.
73. Wu G, Mannam AP, Wu J et al. Hypoxia induces myocyte-dependent COX-2 regulation in endothelial cells: Role of VEGF. Am J Physiol Heart Circ Physiol 2003; 285(6): H2420–9.
74. Risau W. Mechanisms of angiogenesis. Nature 1997; 386(6626): 671–74.
75. Bikfalvi A, Klein S, Pintucci G, Rifkin DB. Biological roles of fibroblast growth factor-2. Endocr Rev 1997; 18(1): 26–45.
76. Cartwright JE, Johnstone AP, Whitley GS. Endogenously produced nitric oxide inhibits endothelial cell growth as demonstrated using novel antisense cell lines. Br J Pharmacol 2000; 131(1): 131–7.
77. Norrby K. Constitutively synthesized nitric oxide is a physiological negative regulator of mammalian angiogenesis mediated by basic fibroblast growth factor. Int J Exp Pathol 2000; 81(6): 423–7.
78. Phillips PG, Birnby LM, Narendran A, Milonovich WL. Nitric oxide modulates capillary formation at the endothelial cell-tumor cell interface. Am J Physiol Lung Cell Mol Physiol 2001; 281(1): L278–90.
79. RayChaudhury A, Frischer H, Malik AB. Inhibition of endothelial cell proliferation and bFGF-induced phenotypic modulation by nitric oxide. J Cell Biochem 1996; 63(2): 125–34.
80. Silveira LR, Pereira-Da-Silva L, Juel C, Hellsten Y. Formation of hydrogen peroxide and nitric oxide in rat skeletal muscle cells during contractions. Free Radic Biol Med 2003; 35(5): 455–64.
81. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 1987; 162: 156–59.