Contraction induced secretion of VEGF from skeletal muscle cells is mediated by adenosine

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Abstract

The role of adenosine and contraction for secretion of VEGF in skeletal muscle was investigated in human subjects and rat primary skeletal muscle cells. Microdialysis probes were inserted into the thigh muscle of seven male subjects and dialysate was collected at rest, during infusion of adenosine and during knee extensor exercise. The dialysate was analyzed for content of VEGF protein and adenosine. The mechanism of VEGF secretion from muscle cells in culture was examined in resting and electro stimulated cells, and in response to the adenosine analogue NECA, and the adenosine A2A receptor specific analog CGS21680. Adenosine receptors A1, A2A and A2B were blocked with DPCPX, ZM241385 and enprofylline, respectively. cAMP dependent protein kinase A (PKA) and Mitogen activated protein kinase (MAPK) were inhibited by H-89 and PD 98509, respectively. The human experiment showed that adenosine infusion enhanced (P<0.05) the interstitial concentration of VEGF protein ~ 4-fold above baseline. Exercise increased (P<0.05) the interstitial VEGF concentration ~6-fold above rest in parallel with a ~3-fold increase in adenosine concentration. In accordance, in cultured muscle cells, NECA and contraction caused secretion of VEGF (p<0.05). The contraction induced secretion of VEGF was abolished by the A2B antagonist enprofylline and by inhibition of PKA or MAPK. The results demonstrate that adenosine causes secretion of VEGF from human skeletal muscle cells and that the contraction induced secretion of VEGF protein is partially mediated via adenosine acting on A2B adenosine receptors. Moreover, the contraction induced secretion of VEGF protein from muscle is dependent on both PKA and MAPK activation, but only the MAPK pathway appears to be adenosine dependent, revealing involvement of additional pathways in VEGF secretion.
Introduction

Vascular endothelial growth factor (VEGF) is believed to be an essential component in capillary growth in skeletal muscle as also evidenced by markedly reduced capillarization in skeletal muscle of animals with a targeted deletion of VEGF in skeletal muscle (Tang et al. 2004). In human skeletal muscle, VEGF is primarily located within the skeletal muscle cells (Jensen et al. 2004a, c) and upon muscle contraction gene-expression (Breen et al. 1996, Gustafsson et al. 1999; Jensen et al. 2004b) as well as the level of interstitial VEGF (Hoffner et al. 2003; Jensen et al. 2004a; Gavin et al. 2007) increases markedly. The primary source of the muscle interstitial VEGF levels is likely to be skeletal muscle cells as electro stimulation of cultured skeletal muscle cells, leads to secretion of VEGF (Jensen et al. 2004c). It is therefore believed that skeletal muscle cells hold an important role in the regulation of capillary growth by secretion of VEGF to the extracellular space where it can act on the vascular endothelial cells. The mechanisms underlying VEGF secretion from skeletal muscle cells have, nevertheless, not been well investigated.

We have previously shown that skeletal muscle contraction leads to formation of adenosine on the surface of skeletal muscle and endothelial cells via the enzyme ecto 5’ nucleotidase (Hellsten & Frandsen 2000; Lynge et al. 2001), and adenosine concentrations have been found to increase in the human skeletal muscle interstitium during exercise (Hellsten et al. 1998, Frandsen et al. 2000; Langberg et al. 2002). Several previous studies have shown that adenosine promotes the expression (Gu et al. 1999), as well as secretion (Jensen et al. 2004c, Adair 2005a, Ryzhov et al. 2007), of VEGF in different cells. In the current study we investigated the role of adenosine in promoting VEGF secretion in skeletal muscle and the role of adenosine as a mediator of contraction.
induced VEGF secretion from skeletal muscle cells. As both cAMP dependent protein kinase A (PKA) and Mitogen activated protein kinase (MAPK) have been implicated in the response to adenosine in cells (d'Angelo et al. 1997; Grant et al. 2001; Klettner and Roider 2009) we also investigated the involvement of these downstream signalling pathways for VEGF secretion. Two experimental models were used: firstly, to determine whether adenosine causes the secretion of VEGF from human muscle in vivo, microdialysis probes were inserted into the thigh muscle of young healthy human subjects and VEGF was determined in the muscle dialysates during basal conditions, during adenosine infusion and, for comparison, during exercise. Secondly, to examine signalling pathways involved in the secretion of VEGF from muscle cells, experiments were performed on cultured rat primary skeletal muscle cells.

Methods

Materials

Dulbecco’s modified Eagle medium (DMEM), fetal calf serum (FCS), horse serum (HS), Dulbecco’s Phosphate buffered saline (DPBS), penstrep (penicillin [10 000 U/mL], streptomycin [10 000 U/mL]), and trypsin were all from Life Technologies. Human umbilical vein endothelial cells in the primary culture stage (HUVEC) and medium 200 with Low Serum Growth Supplement (LSGS) containing fetal bovine serum, fibroblast growth factor, heparin and epidermal growth factor were from Cascade Biologics Inc, Portland, OR). DNAse, trypsin/EDTA solution, glucose, S'-N-ethylcarbox- amidoadenosine (NECA), enprofylline, and CGS21680 (4-[2-[[6-Amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H -purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride) were all products from Sigma, MO, USA. Collagenase (type II) was from Worthington Biochemicals. TriReagent
from the Molecular Research Center (OH, USA). PD 98509 was from Calbiochem. 4-(2-[[7-
96 Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl]phenol (ZM 241385), 8-
97 Cyclopentyl-1,3-dipropylxanthine(DPCPX), and N-[2-[[3-(4-Bromophenyl)-2-
98 propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89) were from
99 TOCRIS Biosciences, UK.

Experimental protocol- microdialysis

Seven healthy, habitually active male subjects with a mean age of 25 (range: 20-38) years,
participated in the study. The subjects were asked not to exercise or ingest caffeine 24
hours prior to the experimental days. Food intake was restricted throughout the experiment
until after the microdialysis sampling where the subjects received a light lunch.

Sampling of the muscle interstitial fluid was performed by inserting four microdialysis
probes into the thigh muscle (vastus lateralis) of the subjects. Sixty minutes after insertion
of the probes, the subjects performed a 10 min exercise bout at 10 Watt to minimize the
tissue response to insertion trauma (Nordsborg et al. 2003). To re-establish resting
conditions, the subjects rested for another 30 min, and the microdialysis sampling protocol
was initiated by collection of dialysate for 2 x 20 min at rest. Adenosine was then infused
at two concentrations, 125 and 1250 μM in two of the probes. The position of these two
probes out of the four was randomized. Each concentration was infused for 30 min.

Dialysate was collected throughout the infusion periods. After the infusion protocol,
dialysate from probes not previously perfused with adenosine was collected at rest and for
30 min during each of 10 and 30 W of exercise. The two bouts were performed
consecutively. All dialysate samples were immediately weighed and frozen in -80°C until
further analysis.
Microdialysis procedure: The semi-permeable fibres used to construct the microdialysis probes had a molecular mass cut-off and inner/outer diameter of 960 kDa and 0.34/0.44 mm (Asahi Medical, Tokyo, Japan), respectively. The probes were made by gluing each end of a fibre 2 cm into a hollow nylon tube (0.50 mm inner diameter and 0.63 mm outer diameter; Portex SIMS, Kent, UK).

Prior to the insertion of the microdialysis probes into the m. vastus lateralis, the skin, subcutaneous tissue and fascia close to both the insertion and exit points were anaesthetised with lidocaine (Xylocaine; 20 mg ml$^{-1}$). Using a 17G/45 mm Venflon I.V. catheter, four microdialysis probes were inserted, ~3-4 cm apart. The direction of the microdialysis probes was aligned with the direction of the muscle fibre. The microdialysis probes were perfused with phosphate buffered saline, pH 7.4, with a high-precision syringe pump (CMA 102, Carnegie Medicine, Solna, Sweden) at a rate of 5 µl min$^{-1}$. A small amount (3.1nM) of [2-$^{3}$H] labelled adenosine was included in the perfusate for the calculation of probe recovery. The main purpose of this determination of probe recovery was to correct for differences in recovery from rest to exercise. Suction was connected to the outflow tube of the 960 kDa probes to prevent fluid loss from the probe during the experiment (Hoffner et al. 2003).

The relative loss for each probe was determined according to the internal reference method (Scheller & Kolb, 1991; Jansson et al., 1994) for [2-$^{3}$H] Ado. Probe recovery (PR) was calculated as PR = [(dpm perfusate – dpm dialysate)/dpm perfusate], where dpm denotes disintegrations per minute. The $^{3}$H activity was measured in a liquid scintillation counter (Tri-Carb 2000; Copenhagen, Denmark) after addition of the perfusate or dialysate...
(5 µl of each) to 3.0 ml of Ultima Gold scintillation liquid (Packard Instruments, Groningen, The Netherlands).

**Primary skeletal muscle cell cultures**

Skeletal muscle cell cultures were prepared from male Wistar male rats (M&B, Denmark). Rats weighing 100 g were sacrificed by cervical dislocation. Carefully the muscle fascia was removed and soleus, gastrocnemius and quadriceps femoris muscles were removed and placed on ice in DPBS with 1% glucose+ 1% penstrep. The muscle tissue was minced into small pieces with scissors and then digested with 0.2% collagenase II in DMEM containing 1% penstrep, for 1.5 hour at 37°C with rotation. After centrifugation at 200 x g for 15 minutes pellet was incubated with rotation in a solution of 0.2% collagenase, 0.01% DNAase and 0.25% trypsin in DMEM containing 1% penstrep for 30 minutes at 37°C. The cells were suspended in primary growth medium (PGM) (DMEM supplemented with 1% penstrep, HS (10%) and FCS (10%)), counted and seeded out onto 35 mm dishes (~ 30 \cdot 10^4 cells per dish) coated with 1% matrigel and incubated at 8% CO₂ and 37 °C. The cells were not passaged, and after 2 days PGM was changed to primary fusion medium (FM) (DMEM supplemented with L-glutamine [2 mM] and HS [10%]) and after 7 additional days the primary skeletal muscle cells were ready for experiments. At this time most of the myocytes have differentiated into multinucleated myotubes and can easily be identified as muscle cells. A small fraction of fibroblasts is present in these cultures but due to their low amount relative to the muscle fibers their contribution is considered negligible.

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).
**Cell culture experiments**

Before experiments were performed, the cell medium was changed to DMEM media containing 0.1% bovine serum albumin (BSA) and the cells were incubated in this media for 16 hours.

*Effect of NECA:* To verify the role of adenosine for VEGF secretion from muscle cells, NECA (1 or 10 μM) was added to muscle cells. Medium for determination of VEGF was collected from the NECA stimulated cells and from non-treated control cells after 24 h of incubation.

*Effect of Muscle contraction:* For the analysis of VEGF secretion with muscle contraction, skeletal muscle cells were electro-stimulated for 2 hours at 50 Hz, 0.6s/0.4s trains, 1 ms pulse width and 10 V. The extracellular medium was collected 24 hours after end of stimulation and medium from non-stimulated control cells were obtained at the same time.

*Role of adenosine receptors for contraction induced VEGF secretion*

To determine the role of adenosine for the contraction induced secretion of VEGF, experiments were also conducted in which the adenosine A₁, the A₂A and the A₂B receptors were inhibited by use of the antagonists DPCPX (50nM), ZM 241385 (50 nM), or enprofylline (100 μM), respectively. The inhibitors were added 30 min prior to electro-stimulation.

In addition, the adenosine A₂A receptor agonist CGS21680 (10 μM) was added to the cells to further examine the role of this receptor for VEGF secretion.

*Role of cAMP dependent PKA and MAPK for the contraction induced secretion of VEGF*
The role of PKA and MAPK for VEGF secretion was examined by addition of H-89 (10 μM) or PD 98509 (150 μM) respectively, 30 min prior to muscle contraction.

**Measurements of VEGF**

The concentration of VEGF protein in the muscle dialysate was analysed by a Quantikine enzyme-linked immunosorbent assay kit (R&D Systems, UK) according to the manufacturer’s protocol. The VEGF assay has the potential to recognise all variants of VEGF-A, but has only been tested with VEGF 121 and 165 and recombinant human VEGF (R&D Systems, UK).

**Statistical analyses**

A one way analysis of variance (ANOVA) for repeated measures was used to evaluate effect of adenosine infusion or exercise on muscle interstitial levels of VEGF and adenosine. Values from cells treated with inhibitors were expressed as fold change relative to control values, which were set to 1. Effect of inhibitors on the 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. Data are presented as means +/- SEM. A value of $P < 0.05$ was accepted as statistically significant.

**Results**

*Microdialysis in human skeletal muscle*

The concentration of muscle interstitial VEGF at rest was 70 pg/ml. Infusion of 125 or 1250 μM adenosine into the skeletal muscle interstitium via microdialysis probes enhanced (p<0.05) the interstitial VEGF concentration ~2.5 and ~ 4-fold, respectively (n= 7; Fig. 1a)
Exercise performed at either 10 or 30 W enhanced the VEGF level six-fold from 85±10 pg/ml at rest (p<0.05; Fig. 1b). The interstitial adenosine concentration was higher at 30 W of exercise than at rest (p<0.05; n=6; Fig. 1c).

Experiments on skeletal muscle cells
Electrostimulation of skeletal muscle cells increased the level of VEGF in the medium and the effect was dependent on the intensity of contraction where stimulation at 1 V and 10 V lead to an increase (p<0.05) in VEGF levels in the medium of 1330 and 2850 pg/ml, respectively (Fig. 2a; n=6).

The non-specific adenosine receptor agonist NECA increased (p<0.05) the secretion of VEGF from muscle cells in a concentration dependent manner (Fig. 2b; n=6).

Adenosine receptor antagonists
None of the used adenosine antagonists, i.e. DPCPX, ZM241385 or enprofylline, affected the basal secretion of VEGF from the muscle cells (data not shown). The stimulation induced increase in VEGF secretion was abolished by enprofylline (p<0.05) but not by ZM 241385 (Fig. 3a-c; n=5). Addition of DPCPX prior to stimulation lowered (P<0.05), but did not completely abolish, the contraction induced increase in VEGF secretion. Addition of the adenosine A$_{2A}$ receptor agonist CGS21680 did not influence VEGF release (Fig. 4; n=10).

Inhibition of PKA and MAPK
Contraction induced secretion of VEGF was abolished (p<0.05) by either inhibition of PKA by H-89 or by MAPK inhibition by PD 98509. Combined inhibition of PKA and MAPK did
not result in an additive inhibitory effect (Fig. 5; n=5). NECA induced secretion of VEGF was abolished by PD 98509 but enhanced by H-89 (P<0.05; Fig. 6; n=5). Combined inhibition with PD98509 and H-89 resulted in a similar VEGF secretion as NECA alone. Addition of H-89 or PD98509 had no effect on the basal level of VEGF secretion.

Discussion

The main findings of the present study were that adenosine causes the secretion of VEGF from human skeletal muscle and that contraction induced VEGF secretion is partly dependent on adenosine receptor activation. The A$_{2B}$ receptor appears to be important for the adenosine mediated effect. Moreover, inhibition of PKA and MAPK both reduced the contraction induced secretion of VEGF, suggesting an involvement of both pathways, although only MAPK appeared to be involved in the adenosine mediated VEGF secretion.

Skeletal muscle cells contain large amounts of VEGF that can be secreted in response to muscle contraction (Hoffner et al. 2003, Jensen et al. 2004) and the resulting increase in muscle extracellular VEGF is believed to be important for the regulation of angiogenesis (Brown & Hudlicka 2003; Egginton 2008). In the current study we investigated contraction induced VEGF secretion and the potential involvement of adenosine. Infusion of adenosine into human skeletal muscle via microdialysis probes enhanced the extracellular level of VEGF up to ~4-fold, whereas an approximate 6-fold increase in VEGF, concomitant with a ~3-fold increase in interstitial adenosine, was observed during muscle contraction. The fact that VEGF secretion in response to adenosine was smaller than that induced by contraction suggests that, additional factors promote VEGF secretion from
muscle cells, or potentially endothelial cells. This was also supported by the finding that the contraction induced secretion of VEGF was reduced by inhibition of PKA whereas NECA induced VEGF secretion was not, suggesting at least two separate pathways.

The observation that adenosine promotes secretion of VEGF from cells in muscle tissue \textit{in vivo}, is in accordance with findings in a previous study by Adair and colleagues (2005b) which showed that venous infusion of adenosine enhanced plasma concentrations of VEGF. Based on our studies on muscle cells in culture, we believe that the increase in muscle interstitial VEGF originates primarily from skeletal muscle cells, although vascular endothelial cells could also be a source. In the study by Adair et al. (2005b) however, it may be assumed that the increase in plasma VEGF was primarily due to release from vascular cells, as we have observed that arterially infused adenosine does not cross over to the interstitium (Nyberg, Hellsten and Mortensen, unpublished results). Combined, the current results and the observation by Adair et al. (2005b) suggest that adenosine is a regulator of VEGF secretion both from skeletal muscle cells and endothelial cells in humans \textit{in vivo}.

It may be argued that the adenosine concentration infused through the microdialysis probe was non-physiological as the levels of adenosine measured were in the nanomolar range. However, considering that the perfusion rate of the microdialysis probe is only 5 \( \mu l/min \), that the interstitial volume is large, and that the half life of adenosine is only a few seconds due to uptake into muscle cells as well as enzymatic degradation (Lynge et al. 2001), the actual concentration in the interstitium may well be in the nanomolar range. Also, when NECA was added to skeletal muscle cells in culture at 1 and 10 \( \mu m \), the magnitude of increase in VEGF secretion was similar to that observed with electro stimulation at 1 and 10 V. This observation supports the notion of a substantial loss of
adenosine in the interstitium to levels far below those used in the perfusate. For apparent reasons the level of adenosine in the interstitium cannot be measured in probes perfused with adenosine.

Adenosine infusion into the muscle interstitium could, in theory, have lead to an enhancement in microcirculatory blood flow. Such an effect would underestimate the effect of adenosine on VEGF secretion as enhanced flow would increase the diffusion gradient between the interstitium and blood and, thereby, removal. However, the influence of infused adenosine on flow was probably limited given the low flow rate of the perfusate in combination with the short half-life of adenosine, as also discussed above.

To determine the importance of adenosine for contraction induced secretion of VEGF from skeletal muscle cells, cultured muscle cells were stimulated with and without adenosine receptor blockade. Electro-stimulation enhanced the extracellular VEGF level by about 50%. Addition of enprofylline prior to electro-stimulation abolished the contraction induced VEGF secretion whereas addition of ZM241385 did not have an effect. Addition of DPCPX partially lowered the contraction induced secretion of VEGF. These observations demonstrate that adenosine is important for the regulation of VEGF secretion from contracting muscle and suggests that A2B may be the primary receptor responsible, although the A1 receptor may also be involved. Moreover the adenosine A2A receptor agonist CGS 21680 had no effect on the secretion of VEGF. The finding of the adenosine A2B receptor being important for VEGF secretion is in accordance with our previous observation that the adenosine A2B receptor is the main mediator of the adenosine induced increase in cAMP in skeletal muscle cells (Lynge et al. 2003). The A2B receptor has previously been described to be of importance in angiogenesis by promoting proliferation and migration of retinal endothelial cells (Grant et al. 2001).
In the current study, we also examined if the MAPK and PKA signalling pathways were involved in contraction induced secretion of VEGF from muscle cells. Both MAPK and PKA inhibition abolished the contraction induced secretion of VEGF from the cells but a combined inhibition of the two blockers did not result in an additive effect. In contrast, the NECA induced VEGF secretion was markedly reduced by MAPK inhibition whereas PKA inhibition, on the contrary, enhanced the VEGF secretion. This effect of NECA is in accordance with that previously observed in endothelial cells where NECA induced activation of the adenosine A2B receptor lead to an activation of MAPK but not PKA (Grant et al. 2001). In the study by Grant and co-workers (2001) PKA was found to be activated by the adenosine A2A receptor, which in skeletal muscle, does not appear to be of importance for adenylate cyclase activation (Lynge et al. 2003) or for VEGF secretion, as shown in the present study by lack of effect of the A2A agonist CGS 21680 on VEGF secretion. Opposing effects of PKA and MAPK have previously been reported; in a study on capillary endothelial cells PKA activation was shown to inhibit MAPK dependent cell proliferation (d’Angelo et al. 1997). Therefore, it would appear that contraction induced secretion of VEGF is partially dependent on adenosine acting on adenosine A2B receptors leading to MAPK activation and partially mediated by PKA activation, independent of adenosine.

Of note was also that, whereas MAPK inhibition lowered VEGF secretion below baseline, MAPK inhibition during NECA stimulation did not. Moreover, MAPK inhibition had no effect on base-line VEGF secretion. Therefore, the effect of PD98509 appeared to be specific to electro stimulated cells. This is an interesting discrepancy that supports that the contraction induced secretion of VEGF also involved other pathways than the adenosine stimulated pathway.
In the human experiment, knee extensions performed at two exercise intensities, enhanced the interstitial VEGF levels, with the VEGF levels being similar at the two intensities. The lack of association to exercise intensity in vivo suggests that VEGF release in response to exercise may be an all or nothing response, which is also supported by our previous finding that passive movement results in a release of VEGF from muscle in vivo that is similar in magnitude to that observed during active exercise (Hellsten et al. 2008). It should, nevertheless, be mentioned that in the skeletal muscle cell-cultures, electro-stimulation at 10 V resulted in a greater extracellular level of VEGF than stimulation at 1 V. The reason for this discrepancy is unclear, but may suggest that in vivo there are additional factors controlling VEGF secretion compared to in isolated cells.

In conclusion, this study demonstrates that adenosine causes the secretion of VEGF from human skeletal muscle cells and that the contraction induced secretion of VEGF is partially mediated via adenosine acting on the adenosine A$_{2B}$ receptor. Moreover, PKA and MAPK activation are involved in the contraction induced secretion, where only the MAPK pathway appears to involve adenosine. It is proposed that adenosine mediated VEGF release is one out of two or several pathways of contraction induced VEGF release.

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References


**Figure legends**

**Fig. 1. VEGF and adenosine concentrations in human skeletal muscle interstitial fluid.**

a) Effect of adenosine infusion at two concentrations (125 and 1250 μM, respectively), through microdialysis probes on human skeletal muscle interstitial VEGF levels. Effect of knee extensor exercise at 10 and 30 W on b) VEGF and c) adenosine concentrations in human skeletal muscle interstitial fluid. Samples of skeletal muscle interstitial fluid were collected from microdialysis probes placed in the thigh muscle of healthy young male subjects. * denotes p<0.05; significantly different from control. N=7.

**Fig. 2. Effect of a) muscle contraction and b) NECA on secretion of VEGF from cultured skeletal muscle cells.**

Primary skeletal muscle cells in culture were treated with either a) electrostimulation at 1 or 10 V, to induce contraction or b) the stable adenosine analog NECA at 1 or 10 μM. The concentration of VEGF in the extracellular medium was determined 24 hours after end of treatment. * denotes p<0.05; significantly different from control. N=6.

**Fig. 3. Effect of adenosine receptor blockade by enprofylline, ZM 241385, or DPCPX on contraction induced VEGF secretion from skeletal muscle cells.**

Primary skeletal muscle cells in culture were electrostimulated to induce contraction in the absence and presence of a) the adenosine A2B receptor blocker enprofylline (100μM), b) the adenosine A2A receptor inhibitor ZM 241385 (50nM); c) the A1 adenosine receptor blocker DPCPX (50nM). The concentration of VEGF in the extracellular medium was
determined 24 hours after end of treatment. * denotes p<0.05; significantly different from control; # denotes significantly different from stimulation. Stim= electro stimulation. N=5.

**Fig.4. Effect of the adenosine A<sub>2A</sub> receptor agonist CGS21680 on VEGF secretion from skeletal muscle cells.**

Primary skeletal muscle cells were treated with CGS 21680 (10μM). The extracellular level of VEGF after 24 h was determined and compared to control. * denotes p<0.05; significantly different from control. N=10.

**Fig. 5. Involvement of MAPK and PKA in contraction induced secretion of VEGF from skeletal muscle cells.** Primary skeletal muscle cells in culture were electro stimulated to induce contraction in the absence and presence of the PKA inhibitor H-89 (10μM) or the MAPK inhibitor PD 98509 (150μM). The concentration of VEGF in the extracellular medium was determined 24 hours after end of treatment. * denotes p<0.05; significantly different from control, # denotes p<0.05, significantly different from electro-stimulation. Stim= electro stimulation. N=4.

**Fig. 6. Involvement of MAPK and PKA in NECA induced secretion of VEGF from skeletal muscle cells.** Primary skeletal muscle cells in culture were treated with the adenosine analog NECA (10μM) in the absence and presence of the PKA inhibitor H-89 or the MAPK inhibitor PD 98509 . The concentration of VEGF in the extracellular medium was determined 24 hours after end of treatment. * denotes p<0.05; significantly different from control, # indicates significantly different from NECA. N=5.
Fig. 1a

VEGF (pg/ml)

Control         Adenosine        Adenosine
125 μM             1250 μM

*
Fig. 1b

VEGF (pg/ml)

Rest 10 W 30 W
Fig. 1c

Adenosine (nmol/L)

Rest        10 W        30W

*
Fig. 2a.

![VEGF expression levels in three conditions: Control, 1V, and 10V. The graph shows a significant increase in VEGF expression in the 10V condition compared to the Control and 1V conditions. The asterisk (*) and hash (#) symbols indicate significant differences.]
Fig. 2b.

![Graph showing VEGF levels with control, 1uM NECA, and 10uM NECA treatments. The graph indicates a significant increase in VEGF levels with 10uM NECA compared to control and 1uM NECA treatments.](image-url)
Fig. 3a

![Graph showing VEGF levels relative to control. The graph compares Control, Stim, and Stim + Enproflline. The Stim condition shows a significant increase (*) compared to Control, and the Stim + Enproflline condition shows a significant decrease (#) compared to Stim.](image-url)
Fig. 3b
Fig. 3c
Fig. 4
Fig. 5

The diagram shows the VEGF levels (relative to control) under different treatment conditions: Cont, Stim, Stim + H-89, Stim + PD98509, Stim + H-89 + PD98509. The control group (Cont) shows a baseline level, while the stimulated groups (Stim) show increased VEGF levels. The addition of H-89 or PD98509 further modulates these levels, with some conditions marked by asterisks (*) and hashtags (#) indicating statistical significance.
Fig. 6

![Graph showing VEGF levels relative to control for different treatments: Cont, NECA, NECA + H-89, NECA + PD98509, NECA + H-89 + PD98509. The graph indicates significant differences with asterisks (*) and a hash (#) indicating statistical significance.](image-url)