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

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Hoier B, Olsen K, Nyberg M, Bangsbo J, Hellsten Y. Contraction induced secretion of VEGF from skeletal muscle cells is mediated by adenosine. Am J Physiol Heart Circ Physiol 299: H857–H862, 2010.—The role of adenosine and contraction for secretion of vascular endothelial growth factor (VEGF) in skeletal muscle was investigated in human subjects and rat primary skeletal muscle cells. Microdialysis probes were inserted in the thigh muscle of seven male subjects, and dialysate was collected at rest, during infusions 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 electrostimulated cells and in response to the adenosine analog NECA and the adenosine A2A receptor specific analog CGS-21680. Adenosine receptors A1, A2A, and A2B were blocked with DPCPX, ZM-241385, 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 approximately fourfold above baseline. Exercise increased (P < 0.05) the interstitial VEGF concentration approximately sixfold above rest in parallel with an approximately threefold 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.

vascular endothelial growth factor

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 (27). In human skeletal muscle, VEGF is primarily located within the skeletal muscle cells (17,19), and upon muscle contraction, gene expression (3, 11, 18) and the level of interstitial VEGF (8, 15, 17) increase markedly. The primary source of the muscle interstitial VEGF levels is likely to be skeletal muscle cells, since electrostimulation of cultured skeletal muscle cells leads to secretion of VEGF (19). 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 (12, 22), and adenosine concentrations have been found to increase in the human skeletal muscle interstitium during exercise (7, 13, 21). Several previous studies have shown that adenosine promotes the expression (10) and secretion (1, 19, 25) 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. Because both cAMP-dependent protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) have been implicated in the response to adenosine in cells (5, 9, 20), we also investigated the involvement of these downstream signaling pathways for VEGF secretion. Two experimental models were used: first, to determine whether adenosine causes the secretion of VEGF from human muscle in vivo, microdialysis probes were inserted in 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. Second, to examine signaling 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), 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 and medium 200 with low serum growth supplement containing FBS, fibroblast growth factor, heparin, and epidermal growth factor were from Cascade Biologics (Portland, OR). DNase, trypsin/EDTA solution, glucose, S’-N-ethylcarbox-amidoadenosine (NECA), enprofylline, and CGS-21680 [4-[2-[(6-amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzene-propanoic acid hydrochloride] were all products from Sigma (St. Louis, MO). Collagenase (type II) was from Worthington Biochemicals. TriReagent was from the Molecular Research Center. PD-98509 was from Calbiochem. 4-[2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl] phenol (ZM-241385), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and N-2-[3-(4-bromophenyl)-2-
prochiralaminoethyl]-S-isooquinolinesulfonamide dihydrochloride (H-89) were from TOCRIS Biosciences.

**Experimental Protocol: Microdialysis**

Seven healthy, habitually active male subjects with a mean age of 25 (range: 20–38) yr participated in the study. The subjects were informed of the risks and discomforts associated with the experiments before giving their informed, written consent to participate. The study was approved by the Ethics Committee of Copenhagen and Frederiksborg and conducted in accordance with the guidelines of the Declaration of Helsinki. The subjects were asked not to exercise or ingest caffeine 24 h before 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 in the thigh muscle (vastus lateralis) of the subjects. After insertion of the probes (60 min), the subjects performed a 10-min exercise bout at 10 W to minimize the tissue response to insertion trauma (24). To reestablish resting conditions, the subjects rested for another 30 min, and the microdialysis sampling protocol was initiated by collection of dialysate for 2 × 20 min at rest. Adenosine was then infused at two concentrations, 125 and 1,250 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 semipermeable fibers 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 fiber 2 cm in a hollow nylon tube (0.50 mm inner diameter and 0.63 mm outer diameter; Portex SIMS, Kent, UK).

Before the insertion of the microdialysis probes in the vastus lateralis, the skin, subcutaneous tissue, and fascia close to both the insertion and exit points were anesthetized with lidocaine (Xylocaine; 20 mg/ml). With the use of a 17-gauge/45-mm Venflon intravenous catheter, four microdialysis probes were inserted, 3–4 cm apart. The direction of the microdialysis probes was aligned with the direction of the muscle fiber. The microdialysis probes were perfused with PBS, pH 7.4, with a high-precision syringe pump (CMA 102; Carnegie Medicine, Solna, Sweden) at a rate of 5 μl/min. A small amount (3.1 nM) of [2-3H]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 (15).

The relative loss for each probe was determined according to the internal reference method (16, 26) for [2-3H]adenosine. Probe recovery (PR) was calculated as PR = [(dpm perfusate - dpm dialysate)/dpm perfusate], where dpm denotes disintegrations per minute. The 3H activity was measured in a liquid scintillation counter (Tri-Carb 2000; Copenhagen) 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 rats (M & B). Rats weighing 100 g were killed 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 h at 37°C with rotation. After centrifugation at 200 g for 15 min, the 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%)], counted, seeded out on 35-mm dishes (~30 × 106 cells/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 [DMEM supplemented with 1-g-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, because to their low amount relative to the muscle fibers, their contribution is considered negligible.

**Cell Culture Experiments**

Before experiments were performed, the cell medium was changed to DMEM media containing 0.1% BSA, and the cells were incubated in this media for 16 h.

**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 nontreated control cells after 24 h of incubation.

**Effect of muscle contraction.** For the analysis of VEGF secretion with muscle contraction, skeletal muscle cells were electrostimulated for 2 h at 50 Hz, 0.6 s/0.4 s trains, 1 ms pulse width, and 10 V. The extracellular medium was collected 24 h after the end of stimulation, and medium from nonstimulated control cells was 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 (50 nM), ZM-241385 (50 nM), or enprofylline (100 μM), respectively. The inhibitors were added 30 min before electrostimulation.

In addition, the adenosine A2A receptor agonist CGS-21680 (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-98059 (150 μM), respectively, 30 min before muscle contraction.

**Measurements of VEGF**

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

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Statistical Analyses

A one-way ANOVA for repeated measures was used to evaluate the effect of adenosine infusion or exercise on muscle interstitial levels of VEGF and adenosine. Values from cells treated with inhibitors were expressed as the degree of change relative to control values, which were set to one. The 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 ± SE. 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 in 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 sixfold 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 and 10 V led to an increase ($P < 0.05$) in VEGF levels in the extracellular medium after 24 h of treatment ($*P < 0.05$, significantly different from control; $n = 6$; Fig. 2A).

The nonspecific adenosine receptor agonist NECA increased ($*P < 0.05$) the secretion of VEGF from muscle cells in a concentration-dependent manner ($n = 6$; Fig. 2B).

Adenosine Receptor Antagonists

None of the used adenosine antagonists, i.e., DPCPX, ZM-241385, 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 ($n = 5$; Fig. 3, A–C). Addition of DPCPX before 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 CGS-21680 did not influence VEGF secretion ($n = 10$; Fig. 4).

**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 ($n = 5$; Fig. 5). NECA-induced secretion of VEGF was abolished by PD-98509 but enhanced by H-89 ($P < 0.05$; $n = 5$; Fig. 6). Combined inhibition with PD-98509 and H-89 resulted in a similar VEGF secretion as NECA alone. Addition of H-89 or PD-98509 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

![Fig. 3. Effect of adenosine receptor blockade by enprofylline, 4-[2-{7-Amino-2-(2-furyl)]1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino}ethyl]phenol (ZM-241385), or 8-cyclopentyl-1,3-dipropylxanthine (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 the adenosine $A_2B$ receptor blocker enprofylline (100 nM; A), the adenosine $A_2A$ receptor inhibitor ZM-241385 (50 nM, B), or the $A_1$ adenosine receptor blocker DPCPX (50 nM; C). The concentration of VEGF in the extracellular medium was determined 24 h after end of treatment. $P < 0.05$, significantly different from control (*) and significantly different from stimulation (#); $n = 5$. Stim, electrostimulation.

![Fig. 4. Effect of the adenosine $A_2A$ receptor agonist 4-(2-{[6-amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino}ethyl)benzenepropanoic acid hydrochloride (CGS-21680) on VEGF secretion from skeletal muscle cells. Primary skeletal muscle cells were treated with CGS-21680 (10 nM). The concentration of VEGF in the extracellular medium was determined 24 h after treatment. $P < 0.05$, significantly different from control (*); $n = 10$.

![Fig. 5. Involvement of mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) in contraction-induced secretion of VEGF from skeletal muscle cells. Primary skeletal muscle cells in culture were electrostimulated to induce contraction in the absence and presence of the PKA inhibitor N-(2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl)-5-isouquinolinesulfonamide dihydrochloride (H-89, 10 μM) or the MAPK inhibitor PD-98509 (150 μM). The concentration of VEGF in the extracellular medium was determined 24 h after the end of treatment. $P < 0.05$, significantly different from control (*) and significantly different from electrostimulation (#); $n = 4$.**
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 (15, 17), and the resulting increase in muscle extracellular VEGF is believed to be important for the regulation of angiogenesis (4, 6). In the current study, we investigated contraction-induced VEGF secretion and the potential involvement of adenosine. Infusion of adenosine in human skeletal muscle via microdialysis probes enhanced the extracellular level of VEGF up to approximately fourfold, whereas an approximate sixfold increase in VEGF, concomitant with an approximately threefold 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 in vivo is in accordance with findings in a previous study by Adair and colleagues (2), 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. (2), however, it may be assumed that the increase in plasma VEGF was primarily due to release from vascular cells, since we have observed that arterially infused adenosine does not cross over to the interstitium (M. Nyberg, Y. Hellsten, and S. P. Mortensen, unpublished results). Combined, the current results and the observation by Adair et al. (2) suggest that adenosine is a regulator of VEGF secretion both from skeletal muscle cells and endothelial cells in humans in vivo.

It may be argued that the adenosine concentration infused through the microdialysis probe was nonphysiological, since the levels of adenosine measured were in the nanomolar range. However, considering that the perfusion rate of the microdialysis probe is only 5 μl/min, that the interstitial volume is large, and that the half-life of adenosine is only a few seconds because of uptake in muscle cells and enzymatic degradation (22), the actual concentration of adenosine in the interstitium during infusion may well be in the nanomolar range. Also, when NECA was added to skeletal muscle cells in culture at 1 and 10 μm, the magnitude of increase in VEGF secretion was similar to that observed with electrostimulation 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 in the muscle interstitium could, in theory, have led to an enhancement in microcirculatory blood flow. Such an effect would underestimate the effect of adenosine on VEGF secretion, since 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. Electrostimulation enhanced the extracellular VEGF level by ~50%. Addition of enprofylline before electrostimulation abolished the contraction-induced VEGF secretion, whereas addition of ZM-241385 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 suggest 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 (23). The A3 receptor has previously been described to be of importance in angiogenesis by promoting proliferation and migration of retinal endothelial cells (9).

In the current study, we also examined if the MAPK and PKA signaling 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 led to an activation of MAPK but not PKA (9). In the study by Grant and coworkers (9), PKA was found to be activated by the adenosine A2A receptor, which in skeletal muscle does not appear to be of importance for adenylyl cyclase activation (23) 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 (5). Therefore, it would appear that contraction-induced secretion of VEGF is partially dependent on adenosine acting on adenosine A2R 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 baseline VEGF secretion. Therefore, the effect of PD-98509 appeared to be specific to electrostimulated 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 (14). It should, nevertheless, be mentioned that, in the skeletal muscle cell cultures, electrostimulation 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 with 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 A2R receptor. Moreover, PKA and MAPK activation is 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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DISCLOSURES

No conflicts of interest are declared by the authors.

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