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Intracellular adenosine formation and release by freshly-isolated vascular endothelial cells from rat skeletal muscle: effects of hypoxia and/or acidosis

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ABSTRACT

Previous studies suggested indirectly that vascular endothelial cells (VECs) might be able to release intracellularly-formed adenosine. We isolated VECs from the rat soleus muscle using collagenase digestion and magnetic-activated cell sorting (MACS). The VEC preparation had >90% purity based on cell morphology, fluorescence immunostaining, and RT-PCR of endothelial markers. The kinetic properties of endothelial cytosolic 5′-nucleotidase suggested it was the AMP-preferring N-I isoform: its catalytic activity was 4 times higher than ecto-5′nucleotidase. Adenosine kinase had 50 times greater catalytic activity than adenosine deaminase, suggesting that adenosine removal in VECs is mainly through incorporation into adenine nucleotides. The maximal activities of cytosolic 5′-nucleotidase and adenosine kinase were similar. Adenosine and ATP accumulated in the medium surrounding VECs in primary culture. Hypoxia doubled the adenosine, but ATP was unchanged; AOPCP did not alter medium adenosine, suggesting that hypoxic VECs had released intracellularly-formed adenosine. Acidosis increased medium ATP, but extracellular conversion of ATP to AMP was inhibited, and adenosine remained unchanged. Acidosis in the buffer-perfused rat gracilis muscle elevated AMP and adenosine in the venous effluent, but AOPCP abolished the increase in adenosine, suggesting that adenosine is formed extracellularly by non-endothelial tissues during acidosis in vivo. Hypoxia plus acidosis increased medium ATP by a similar amount to acidosis alone and adenosine 6-fold; AOPCP returned the medium adenosine to the level seen with hypoxia alone. These data suggest that VECs release intracellularly formed adenosine in hypoxia, ATP during acidosis, and both under simulated ischaemic conditions, with further extracellular conversion of ATP to adenosine.

1. Introduction

Adenosine is an important mediator of skeletal muscle blood flow regulation: plasma adenosine increases during muscle contractions [1,2], ischaemia [3] or systemic hypoxia [4,5]. Intravascular adenosine is unlikely to originate from the interstitial space, firstly because VECs form a substantial barrier to diffusion [5,6], and secondly because plasma adenosine increases while muscle interstitial adenosine remains unchanged during ischaemia or systemic hypoxia [3,4]. Therefore, blood cells or VECs may be possible sources of plasma adenosine. During muscle contraction, elevated plasma ATP might give rise to extracellular adenosine formation [7], but plasma adenine nucleotides were unchanged in hypoxia [5], raising the possibility that intracellularly-formed adenosine had been released. VECs are the most likely source of plasma adenosine during hypoxia or ischaemia, since the arterial concentration was unchanged [5], which rules out release from blood cells, and thromboxane B2 was not increased [3], which rules out platelet aggregation as its source.

It is uncertain whether VECs release adenosine under physiological conditions: acute hypoxia failed to stimulate adenosine release from aortic endothelial cells, although severe ATP depletion elevated intracellular adenosine formation and release [8,9].

Abbreviations: 5′N, 5′-nucleotidase; AD, adenosine deaminase; AK, adenosine kinase; AOPCP, α,β methylene ADP; Km, Michaelis constant; MACS, magnetic-activated cell sorting; PBS, phosphate-buffered saline; VEC, vascular endothelial cell; VEGF, vascular endothelial growth factor; Vmax, maximal activity; vWF, von Willebrand factor.

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Microvascular endothelial cells in primary culture did not accumulate adenosine extracellularly [10] and tracer studies concluded that coronary endothelial cells contributed minimally to adenosine release in vivo [11].

Seven isoforms of 5′-nucleotidase (5′N), have been cloned, including both cytosolic and ecto-forms [12]: only cytosolic 5′N-I (c5′N-I) and ecto-5′N (e5′N) metabolise AMP to adenosine [13], whereas cytoplasmic 5′N-II (c5′N-II) and 5′N-III are IMP- and UMP-preferring, respectively. C5′N-II is expressed ubiquitously, but c5′N-I is reported to be expressed only in heart, brain and pigeon breast muscle [14,15], implying that only these tissues should be capable of intracellular adenosine formation. In heart, ischaemic conditions activate c5′N-I to a level where intracellularly-formed adenosine may be released [16], but in most skeletal muscles c5′N-I has low activity, while adenosine deaminase (AD) and adenosine kinase (AK), can remove adenosine at a higher rate than it is formed [17].

In order to release intracellularly-formed adenosine, VECs would need either a high c5′N-I activity, or else adenosine-removing enzymes with low activity. The capacity of endothelial c5′N for adenosine formation has not been studied. Since endothelial 5′N activity declines in culture [18], and VECs lost the ability to release ATP in response to shear stress by only the second passage [19], we investigated intra- and extracellular adenosine formation during hypoxia or acidosis using VECs freshly-isolated from skeletal muscle. The contributions of c5′N and e5′N to adenosine formation at low pH were further studied in the intact buffer-perfused rat muscle.

2. Materials and methods

2.1. Preparation of animals

All experimental protocols were approved by the University of Hong Kong Committee on the Use of Live Animals in Teaching and Research. Male Sprague–Dawley rats (150–200 g for cell isolation or 400–500 g for muscle perfusion) were anaesthetized with i.p. sodium pentobarbitone (6.0–7.0 mg/100 g; Sagatal, RMB Animal Health Ltd., Dagenham, UK).

2.2. Isolation of VECs and magnetic-activated cell sorting (MACS)

A femoral artery was cannulated distal to the gracilis branch, and perfused for 30 min at 1.5 ml/min with phosphate-buffered saline (PBS; pH 7.4) containing 3% collagenase, 3% BSA, 0.05% trypsin, 0.1 mM EDTA, 50 U/ml penicillin, 50 mg/ml streptomycin and 2 mM L-glutamine, equilibrated with 95% O_2/5% CO_2. The femoral vein was cannulated to allow venous effluent to run to waste.

After sacrificing the rat with an overdose of sodium pentobarbitone, the perfused vessels and soleus muscle were rapidly removed and placed in 4°C PBS containing penicillin (50 U/ml), streptomycin (50 μg/ml) and L-glutamine (2 mM). All subsequent work was performed under careful sterile conditions in a fume hood. VECs were separated from the tissue by further collagenase digestion, and purified first by density gradient centrifugation, then by differential cell attachment, and finally by MACS using DynaBeads and the CELLection Pan Mouse IgG Kit (Dynal-Invitrogen, Lake Success, USA) as previously described [20]. At each stage of the purification process, recovery was assessed by cell counting using a haemocytometer.

2.3. Analysis of purity of VEC suspension

Purity of the VEC suspension was assessed from cell morphology, fluorescence immunostaining, and RT-PCR, and compared to the EA.hy926 endothelial cell line as a positive control.

VECs suspended in fresh, pre-warmed EGM-2-MV were incubated in 6-well plates (Iwaki, Japan) at 37°C with 5% CO_2, rinsed and re-fed with fresh media after 24 h, and thereafter every 2 days. Cultures were monitored daily by phase-contrast microscopy; once the primary culture had grown to confluence, it was photographed for visual assessment of morphology under a Nikon TMS microscope (10×lens, Nikon, Japan).

Fluorescence immunostaining of two endothelial-specific antibodies, CD31 and vWF, was performed in confluent and sub-confluent VECs and in the EA.hy926 endothelial cell line [20] Cells were imaged under epifluorescence using an Olympus Fluoview I×71 confocal microscope (Olympus Optical Co. Ltd., Japan). Purity of the VEC preparation was estimated by visually counting the percentage of immunostained cells.

RNA was extracted for RT-PCR from primary cultured VECs and EA.hy926 cell line, using illustra RNAspin Mini (GE Healthcare, UK Limited, Buckinghamshire, UK). RT-PCR was performed using 3.0 μl × 0.53 μg/μl extracted RNA for primary VECs or 2.0 μl × 0.74 μg/μl extracted RNA for EA.hy926 cells, 0.5 μl RT/Platinum Taq Polymerase Mix at 45 °C for 20 min, and 94 °C for 2 min for reverse transcription. The PCR was carried out at 94 °C, 55 °C and 72 °C for 30 cycles (SuperScript™ One-Step RT-PCR with Platinum® *Taq*, Invitrogen, Carlsbad, CA, USA) using primers for VEGF receptor flt-1, VEGF receptor KDR (flk-1) and eNOS; S-16, a housekeeping gene, was used as the internal control. PCR products were subjected to agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light.

2.4. Activities of adenosine-metabolising enzymes from VECs

VECs were homogenized and separated into membrane and cytosolic fractions by differential centrifugation as described previously [17]. The protein concentrations of the crude homogenate and the separated fractions were determined using the Bio-Rad protein assay, and activities of adenosine-metabolising enzymes were measured as previously described [17]. 5′N was assayed separately in the cytosolic and membrane fractions at 2.5–2000 μM AMP. AD and AK were assayed in the crude homogenate, since the membrane fraction contained negligible amounts of these enzymes, at adenosine concentrations of 100–2000 μM for AD or 0.5–400 μM for AK. Reactions were started by adding 6 μl cell homogenate to 14 μl incubation medium (pH 7.0) and terminated after 15 min incubation at 30°C by addition of 3.5 μl 1.5 M perchloric acid-EDTA. The pH was adjusted to 6.0–7.0 and the mixture was centrifuged at 10,000 × *g* for 15 min to remove precipitate; reaction products were determined in 20 μl of the supernatant using HPLC [5]. 5'N activities of the cytosolic and membrane fractions were also assayed at a single substrate concentration of 200 μM, with the pH of the incubation medium adjusted at 0.5 unit intervals from 6.0 to 8.0.

2.5. Accumulation of adenosine and ATP in the incubation medium of intact VECs

Freshly isolated VECs were seeded in six-well plates (1–9 × 10^6 cells/ml, 2 ml/well) with fresh 2% FBS-enhanced DMEM (pH 7.2–7.4) and exposed to normoxia (20% O_2/5% CO_2), hypoxia (2% O_2/93% N_2/5% CO_2), acidosis (pH 6.0) or hypoxia + acidosis at 37 °C for 24 h. AOPCP (50 μM), an inhibitor of e5'N, was added to half the wells prior to every treatment. After incubation, the cell samples were collected and centrifuged at 1800 × *g* for 10 min; the resulting supernatants (the incubation media) were analyzed by HPLC.

2.6. Appearance of adenosine and AMP in the venous effluent from whole perfused muscle

One gracilis muscle was vascularly isolated and perfused as described previously [17]. The perfusion buffer pH was adjusted

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