Biochemical and Biophysical Research Communications 450 (2014) 93–98

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

G.Y. Le [1], H.C. Essackjee [2], H.J. Ballard *

Department of Physiology, The University of Hong Kong, Pokfulam, Hong Kong

ARTICLE INFO

Article history:
Received 13 May 2014
Available online 24 May 2014

Keywords:
Vascular endothelial cells
Adenosine
5′-Nucleotidase
Hypoxia
Acidosis

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.

* Corresponding author. Address: Department of Physiology, The University of Hong Kong, 21 Sassoon Road, Hong Kong. Fax: +852 28559730.
E-mail address: ballard@hku.hk (H.J. Ballard).
[1] Present address: Department of Physical Therapy and Rehabilitation, University of Minnesota, Minneapolis MN 55455, USA.
[2] Present address: King Saud Bin AbdulAziz University for Health Sciences, College of Basic Sciences, Riyadh, Saudi Arabia.

http://dx.doi.org/10.1016/j.bbrc.2014.05.066
0006-291X/

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

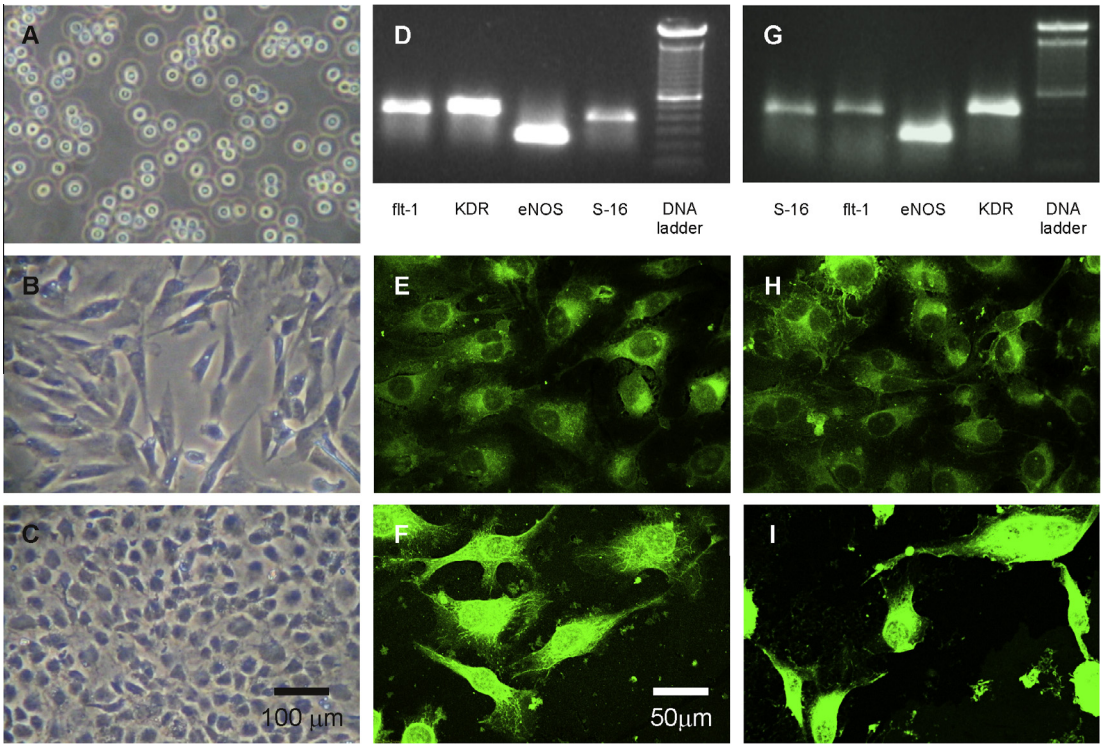

Fig. 1. Comparison of freshly isolated VECs to EA.hy926 endothelial cell line. Phase-contrast microscopy of the freshly isolated VECs in culture after 2 days (A), 1 week (B) or 2 weeks (C). RT-PCR analysis of EA.hy926 (D) and VECs (G). Fluorescence immunostaining for CD31 on EA.hy926 (E) and VECs (H), and for vWF on EA.hy926 (F) and VECs (I).

(if necessary) by changing the rate of bubbling 95% O_2/5% CO_2 through the buffer reservoir; arterial perfusion pressure was recorded as a measure of vascular resistance. Venous effluent pH was monitored with an electrode in a flow-through chamber in the venous cannula. Two 3-min samples of venous effluent were collected over ice during each treatment: samples were centrifuged at 30,000 r.p.m. for 15 s: duplicate 200 μl aliquots were deproteinised with 1.5 ml ice-cold acetone and extracted with 1.5 ml chloroform before analysis by HPLC [5]. The muscle was perfused at pH 7.4 for 10 min, then for a further 10 min using pH 6.8 buffer. This procedure was repeated in a second group of rats using buffers supplemented with 50 μM AOPCP. On completion of the experimental procedures, the rat was killed with an overdose of sodium pentobarbitone.

2.7. Statistical analysis and calculations

Values are the mean ± S.E.M. of 6–14 experiments for intact cell studies, 8–11 experiments for enzymes studies or 6 experiments for buffer-perfused whole muscles. Statistical significance was determined using one-way ANOVA followed by Fisher LSD test. Enzyme kinetic values were determined from the double reciprocal (Lineweaver–Burk) plots of substrate concentration versus activity, in which, the *x*-axis intercept = −1/km and the *y*-axis intercept = 1/Vmax.

3. Results and discussion

3.1. Analysis of purity of VEC suspension

VECs must be separated from other cell types for study; VEC function is best studied in freshly-isolated cells, as the properties of cell lines differ substantially from those of primary cells. VEC recovery from the centrifugation and pre-attachment steps was $6.0 \pm 1.0 \times 10^6$ cells/ml; following MACS, recovery increased to $4.9 \pm 0.5 \times 10^5$ cells/ml. Thus, our isolation procedure increased both yield and purity compared to the previous methods from which it was adapted [20–22]. In culture, the VECs grew to a confluent monolayer in 10–14 days and acquired the characteristic cobblestone morphology for endothelial cells (Fig. 1). Fluorescence immunostaining of endothelial-specific antibodies, CD31 and vWF, showed similar labeling patterns in the isolated VECs and the EA.hy926 cells (Fig. 1). More than 90% of the cells in the VEC preparation gave a positive immunostaining result for CD31 and vWF. RT-PCR found mRNA expression for the VEGF receptors KDR and flt-1, eNOS and the internal positive control, S-16 in both EA.hy926 cell line and freshly-isolated VECs (Fig. 1).

3.2. Activities of adenosine-metabolising enzymes from VECs

VEC c5'N had a significant AMP hydrolyzing activity, with a Vmax of 14.3 nmol/min/mg (Fig. 2): Since c5′N-II had negligible activity under these assay conditions [23], we assume this activity to arise from the c5′N-I isoform. Previously, only heart muscle and pigeon breast muscle have been reported to show a significant cytoplasmic AMP hydrolyzing activity [15,23]. E5'N had a Vmax of 6.5 nmol/min/mg, similar to the value in cultured aortic endothelial cells [24], but less than half that of c5′N,and an order of magnitude less than e5′N of vascular smooth muscle or skeletal muscle [17,25].

The km of c5′N was 247 μM, in good agreement with the value from purified c5'N-I from pigeon breast muscle [15], whereas e5′N had a km of 473 μM, indicating that the substrate affinity of c5′-N was also higher. The Vmax/km ratio, an overall measure of the enzyme's ability to convert substrate into product, was 0.058 for c5′N and 0.014 for e5′N.

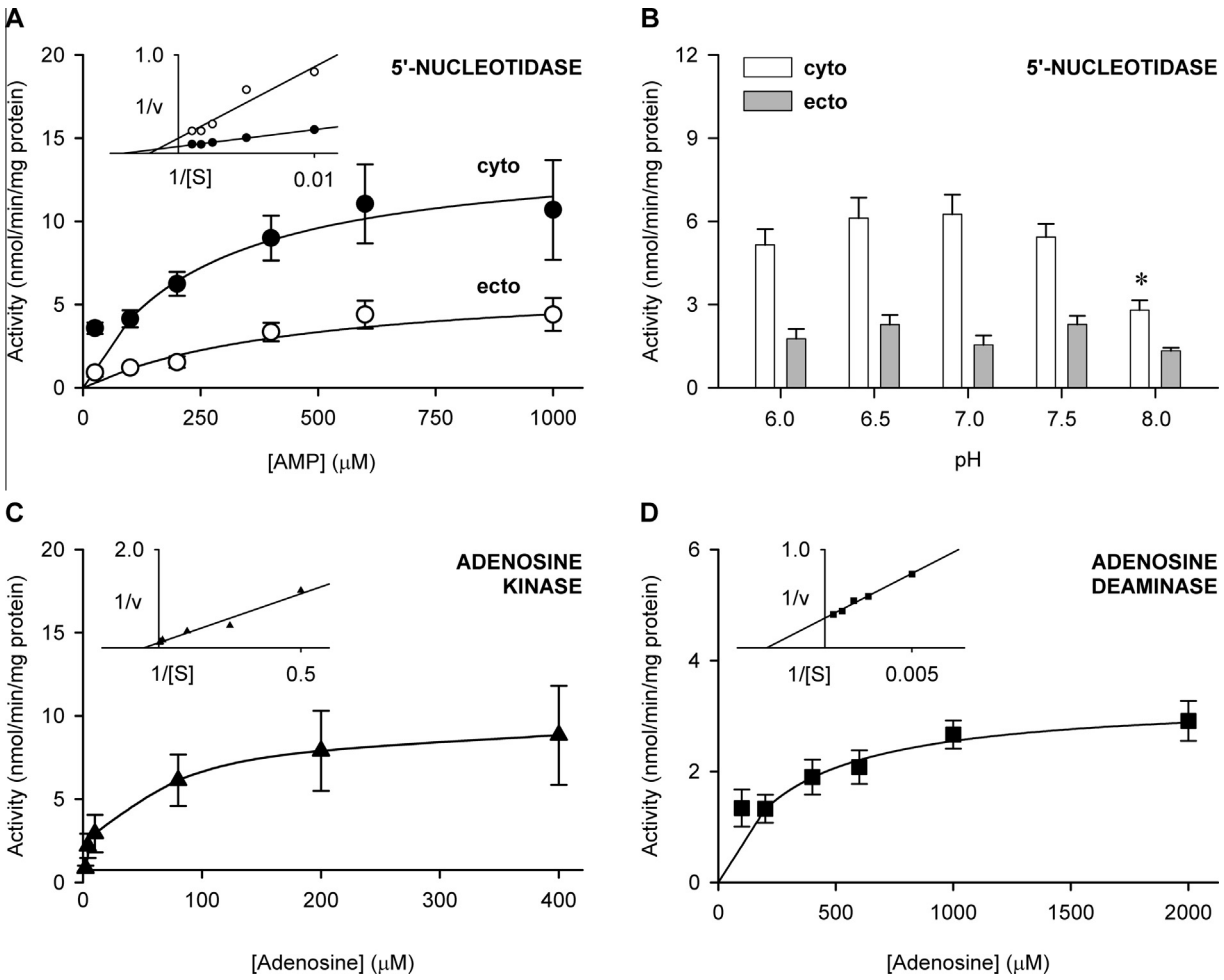

Fig. 2. Activities of adenosine metabolising enzymes from VECs. Michaelis–Menten plots with inset double-reciprocal plots of enzyme activity versus substrate concentration for 5′N (A) in cytosolic (cyto) or membrane (ecto) fractions, and for AK (C) and AD (D) in whole cell homogenate. B, pH dependence of c5′N and e5′N at a substrate concentration of 200 μM. Values are the mean ± S.E.M. of 6 (A, C, D) or 11 (B) estimations, each using a different cell homogenate. *Significantly different from activity in the same preparation at all other pH values.

The optimum pH for c5′N was 7.0 (Fig. 2), similar to c5′N-I in human heart and pigeon breast muscle [23,26]; this further confirms that c5′N-II was not active in our assay as its pH optimum lies at 6.0–6.5 [27]. E5′N activity remained unchanged across the pH range 6.0–8.0 (Fig. 2).

AK had both higher activity and higher substrate affinity than AD (Fig. 2). Thus, the Vmax/km ratio for AK (0.50) was 50 times higher than that for AD (0.01), suggesting that adenosine formed or taken up into VECs would be incorporated into adenine nucleotides, rather than deaminated to inosine. Previous studies using inhibitors or tracers also estimated that 80–90% of adenosine taken up by VECs was incorporated into adenine nucleotides. AK and c5′N had similar Vmax/km ratios, suggesting that adenosine may be incorporated into adenine nucleotides as fast as it is formed.

3.3. Accumulation of adenosine, AMP and ATP in the incubation medium of intact VECs

After 24 h normoxic incubation, the medium surrounding the VECs in primary culture contained 66 ± 15 nM ATP. Hypoxia did not increase medium ATP (Fig. 3), in agreement with previous reports that hypoxia stimulated ATP release from pulmonary VECs [28], but not from systemic VECs [8,19,29].

Acidosis or hypoxia + acidosis produced similar increases in ATP (Fig. 3). AOPCP did not alter ATP under any of the conditions tested. Endothelial cells are known to release ATP in response to shear stress or to various agonists, but this is the first report that acidosis stimulated ATP release from VECs: both connexin 43 and pannexin 1 have been implicated as ATP release channels in vascular endothelia [29,30], whereas shear–stress-induced ATP release is reported to be vesicular [31].

Adenosine was 55 ± 7 nM in control; it was unaffected by acidosis, but increased to 108 nM in hypoxia, and 296 nM in hypoxia + acidosis (Fig. 3). AOPCP did not alter adenosine in normoxia, hypoxia or acidosis, but reduced it in hypoxia + acidosis to a similar level to that in hypoxia alone (Fig. 3), suggesting that hypoxia directly stimulated the release of adenosine from the endothelial cells, whereas acidosis + hypoxia, a condition of simulated ischaemia, stimulated both adenosine and ATP release from VECs, with subsequent extracellular conversion of ATP to adenosine by e5′N.

AMP was 713 ± 83 nM in control. Neither hypoxia nor acidosis significantly altered it; after 24 h of hypoxia + acidosis it was 950 nM (significantly higher than control in paired *t*-test), further supporting the suggestion that some adenosine was formed extracellularly under these conditions.

Previous in vivo studies suggested indirectly that VECs released adenosine during systemic hypoxia: venous adenosine, but not venous ATP, was increased [5]; the increase in femoral vascular conductance was severely blunted by theophylline or 8-phenylt-

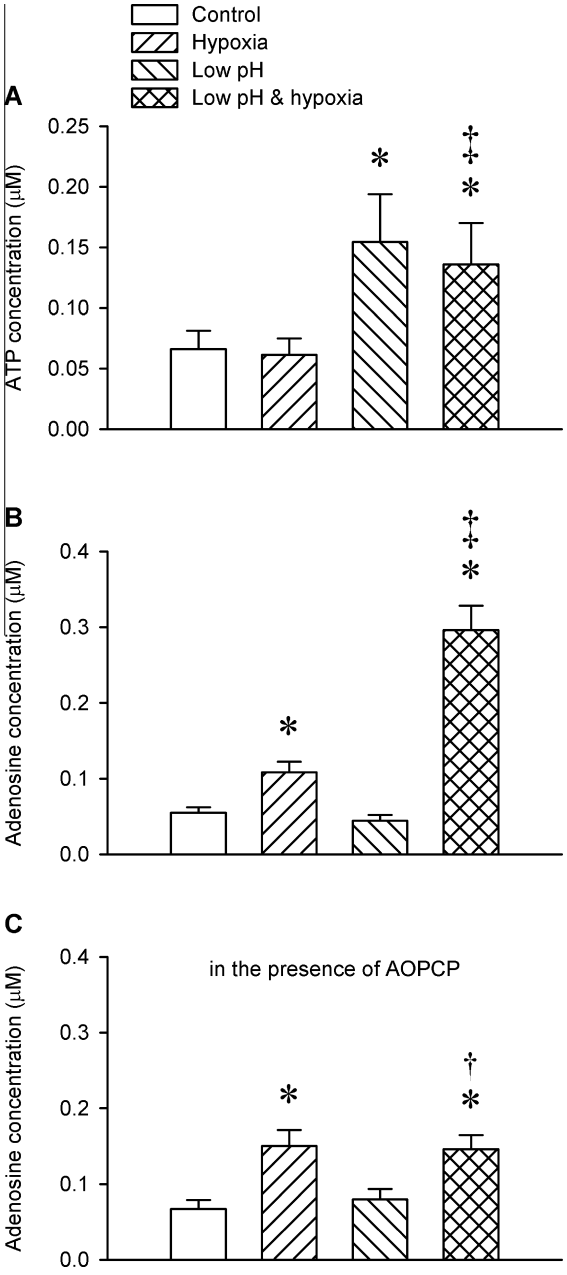

Fig. 3. ATP (A) and adenosine (B and C) concentrations in the extracellular medium of VECs following 24 h incubation. Values are the mean ± S.E.M. of 6–11 estimations. *Significantly different from control; ‡hypoxia plus acidosis significantly different from hypoxia alone; †significant difference between the presence and absence of AOPCP.

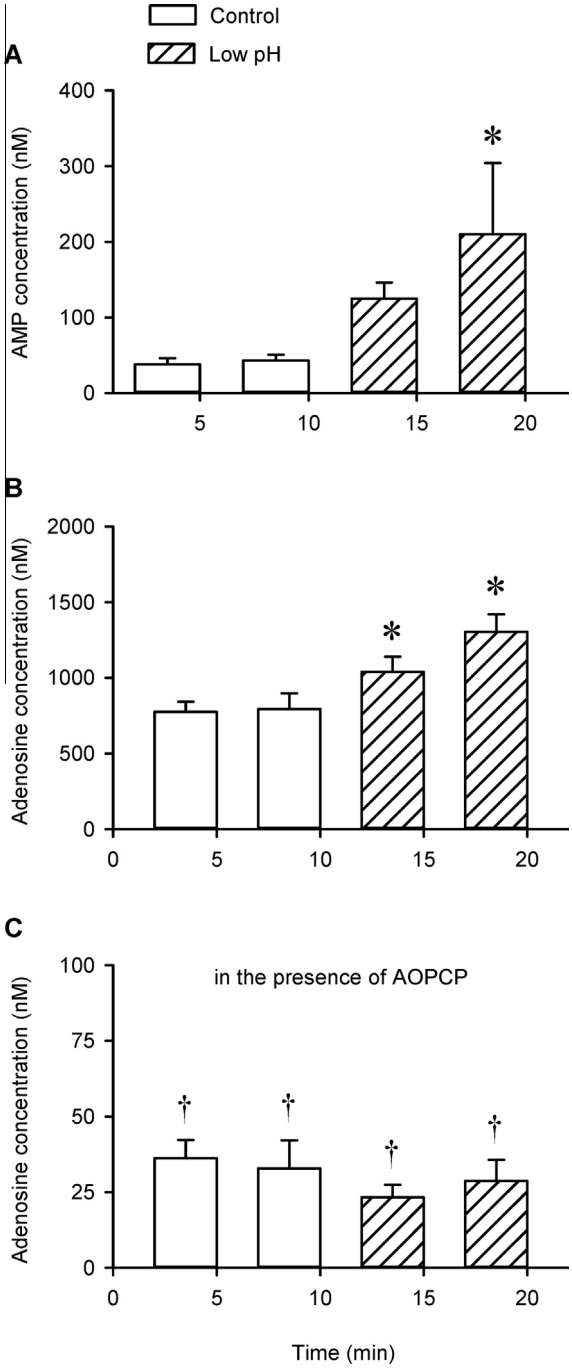

Fig. 4. Concentrations of AMP (A) and adenosine (B and C) in the venous effluent from the isolated gracilis muscle during perfusion with pH 7.4 buffer (control) or pH 6.8 buffer (low pH). Values are the mean ± S.E.M. of 6 estimations in 6 rats. *Significantly different from control; †significantly different from values without AOPCP.

hephylline [32] but unaffected by AOPCP [33], indicating that it was largely mediated by adenosine which was not extracellularly formed. In heart, c5′N-I is strongly activated by ADP [23]: it is possible that cytoplasmic ADP was increased due to ATP breakdown in VECs during hypoxia, which might increase c5′N-I activity, and account for the increased release of intracellularly-formed adenosine.

The failure of acidosis to elevate extracellular adenosine, despite the elevated ATP, suggests that one of the enzymes involved in extracellular conversion of ATP to adenosine may be inhibited at low pH. CD39, the predominant ATPase expressed by VECs [34], can convert either ATP or ADP to AMP, which is then converted to adenosine by e5′N. E5′-N did not exhibit any significant pH dependence (Fig. 4B), but CD39 has a pH maximum at 8 and its activity declines very sharply at low pH [35]. The failure of acidosis to elevate extracellular AMP in our study also suggests that the VEC CD39 was inhibited at low pH.

Addition of a hypoxic stimulus at low pH restored the extracellular conversion of ATP to adenosine, which implies that hypoxia activates an enzyme involved in the ATP to adenosine conversion. Hypoxia increases cell-surface expression and activity of both CD39 and e5′N in VECs [24,36,37], which may account for the extracellular adenosine formation under hypoxia + acidosis conditions.

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

The gracilis muscles were perfused at 43 ± 1.5 μl/min/g; the venous pH was 7.33 ± 0.03 during perfusion with pH 7.4 buffer, and 7.06 ± 0.05 with pH 6.8 buffer. Arterial perfusion pressure decreased from 8.9 ± 0.3 to 6.8 ± 0.1 kPa during pH 6.8 perfusion, and to 7.1 ± 0.3 kPa during pH 6.8 perfusion in the presence of AOPCP.

Venous adenosine and AMP concentrations were 780 ± 60 and 40 ± 5 nM, respectively, at pH 7.4 (Fig. 4), suggesting that the rate of extracellular conversion of AMP to adenosine is much higher in vivo than in the cultured VECs, and therefore that it may involve ectoenzymes expressed on another cell: CD39 is expressed on vascular smooth muscle, and previous studies have shown that vascular smooth muscle had a 17 times higher 5′N activity than endothelium [25].

Perfusion of the muscle with pH 6.8 buffer increased venous adenosine and AMP (Fig. 4), indicating that there is adequate capacity for extracellular conversion of ATP to adenosine under acidotic conditions in vivo. During pH 7.4 perfusion in the presence of AOPCP, venous adenosine decreased (Fig. 4), whereas venous AMP increased to 190 + 13 nM. AOPCP completely abolished the increase in venous adenosine during perfusion with pH 6.8 buffer (Fig. 4), which confirms the findings from the VEC study, namely, that acidosis did not bring about the release of intracellularly-formed adenosine, but rather, increased the extracellular concentration of adenine nucleotides. Extracellularly-formed adenosine contributed to vasodilation during in vivo acidosis, since the decrease in perfusion pressure was attenuated by treatment with AOPCP.

Conclusions

VECs had a relatively high capacity for intracellular adenosine formation, and appeared to express the AMP-preferring c5′N-I, based on the kinetic properties of the enzyme; e5′N activity was low. AK from VECs had both a higher activity and a higher substrate affinity than AD, suggesting that intracellular adenosine would be mainly incorporated into adenine nucleotides. Hypoxia led to the release of intracellularly-formed adenosine from the VECs, whereas acidosis stimulated ATP but not adenosine release; extracellular conversion of ATP to adenosine occurred during acidosis in the perfused muscle in vivo, but not in isolated VECs. Simulated ischaemia (acidosis plus hypoxia) stimulated the release of both adenosine and ATP from the VECs, with significant extracellular formation of adenosine.

Acknowledgments

This work was supported by RGC Direct Allocation grant no. 323-21400-014464-102007333. We are grateful to Mr. M.K. Yip and Mr. C.P. Mok for technical assistance.

References

[1] J.G. Dobson, R.R. Rubio, R.M. Berne, Role of adenine nucleotides, adenosine, and inorganic phosphate in the regulation of skeletal muscle blood flow, Circ. Res. 29 (1971) 375–384.
[2] H.J. Ballard, D. Cotterrell, F. Karim, Appearance of adenosine in venous blood from contracting gracilis muscle and its role in vasodilation in the dog, J. Physiol. 387 (1987) 401–413.
[3] F. Costa, P. Sulur, M. Angel, et al., Intravascular source of adenosine during forearm ischemia in humans: implications for reactive hyperemia, Hypertens 33 (1999) 1453–1457.
[4] S.M. Lo, F.M. Mo, H.J. Ballard, Interstitial adenosine concentration in rat red or white skeletal muscle during systemic hypoxia or contractions, Exp. Physiol. 86 (2001) 593–598.
[5] F.M. Mo, H.J. Ballard, The effect of systemic hypoxia on interstitial and blood adenosine, AMP, ADP and ATP in dog skeletal muscle, J. Physiol. 536 (2001) 593–603.
[6] S. Nees, V. Herzog, B.F. Becker, et al., The coronary endothelium: a highly active metabolic barrier for adenosine, Basic Res. Cardiol. 80 (1985) 515–529.
[7] S. Mortensen, P. Thaning, M. Nyberg, et al., Local release of ATP into the arterial inflow and venous drainage of human skeletal muscle: insight from ATP determination with the intravascular microdialysis technique, J. Physiol. 589 (2011) 1847–1857.
[8] J.C. Shryock, R. Rubio, R.M. Berne, Release of adenosine from pig aortic endothelial cells during hypoxia and metabolic inhibition, Am. J. Physiol. 254 (1988) H223–H229.
[9] R.T. Smolenski, Z. Kochan, R. McDouall, et al., Endothelial nucleotide catabolism and adenosine production, Cardiovasc. Res. 28 (1994) 100–104.
[10] Y. Hellsten, U. Frandsen, Adenosine formation in contracting primary rat skeletal muscle cells and endothelial cells in culture, J. Physiol. 504 (1997) 695–704.
[11] H. Bardenheuer, B. Whelton, H.V. Sparks, Adenosine release by the isolated guinea pig heart in response to isoproterenol, acetylcholine, and acidosis: the minimal role of vascular endothelium, Circ. Res. 61 (1987) 594–600.
[12] V. Bianchi, J. Spychala, Mammalian 5′-nucleotidase, J. Biol. Chem. 278 (2003) 46195–46198.
[13] G.B. Sala-Newby, N.V.E. Freeman, A.C. Skladanowski, A.C. Newby, Distinct roles for recombinant cytosolic 5′-nucleotidase-I and -II in AMP and IMP catabolism in COS-7 and H9c2 rat myoblast cell lines, J. Biol. Chem. 275 (2000) 11666–11671.
[14] G.B. Sala-Newby, A.C. Skladanowski, A.C. Newby, The mechanism of adenosine formation in cells, J. Biol. Chem. 274 (1999) 17789–17793.
[15] K. Tkacz-Stachowska, K. Lechward, A.C. Skladanowski, Isolation and characterization of pigeon breast muscle cytosolic 5′ nucleotidase-I (cN-I), Acta Biochim. Pol. 52 (2005) 789–796.
[16] A. Darvish, P.J. Metting, Purification and regulation of an AMP-specific cytosolic 5′-nucleotidase from dog heart, Am. J. Physiol. 264 (1993) H1528–H1534.
[17] B. Cheng, H.C. Essackjee, H.J. Ballard, Evidence for control of adenosine metabolism in rat oxidative skeletal muscle by changes in pH, J. Physiol. 522 (2000) 467–477.
[18] L.W. Hayes, C.A. Goguen, A.L. Stevens, et al., Enzyme activities in endothelial cells and smooth muscle, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 2531–2535.
[19] P. Bodin, G. Burnstock, Synergistic effect of acute hypoxia on flow-induced release of ATP from cultured endothelial cells, Experientia 51 (1995) 256–259.
[20] C.A. Frye, C.W. Patrick, Isolation and culture of rat microvascular endothelial cells, In Vitro Cell. Dev. Biol. Anim. 38 (2002) 208–212.
[21] P.W. Hewett, J.C. Murray, Immunomagnetic purification of human microvessel endothelial cells using Dynabeads coated with monoclonal antibodies to PECAM-1, Eur. J. Cell Biol. 62 (1993) 451–454.
[22] S. Nistri, L. Mazzetti, P. Failli, D. Bani, High-yield method for isolation and culture of endothelial cells from rat coronary blood vessels suitable for analysis of intracellular calcium and nitric oxide biosynthetic pathways, Biol. Proced. Online 4 (2002) 32–37.
[23] A.C. Skladanowski, R.T. Smolenski, M. Tavenier, et al., Soluble forms of 5′-nucleotidase in rat and human heart, Am. J. Physiol. 270 (1996) H1493–H1500.
[24] S. Ledoux, I. Runembert, K. Koumanov, et al., Hypoxia enhances ecto-5′-nucleotidase activity and cell surface expression in endothelial cells, Circ. Res. 92 (2003) 848–855.
[25] J.D. Pearson, J.S. Carleton, J.L. Gordon, Metabolism of adenine nucleotides by ectoenzymes of vascular endothelial cells, Biochem. J. 190 (1980) 421–429.
[26] A.C. Newby, The pigeon heart 5′-nucleotidase responsible for ischaemia-induced adenosine formation, Biochem. J. 253 (1988) 123–130.
[27] R. Itoh, Purification and some properties of cytosol 5′-nucleotidase from rat liver, Biochim. Biophys. Acta 657 (1981) 402–410.
[28] H.N. Woodward, A. Anwar, S. Riddle, et al., PI3K, Rho, and ROCK play a key role in hypoxia-induced ATP release and ATP-stimulated angiogenic responses in pulmonary artery vasa vasorum endothelial cells, Am. J. Physiol. 297 (2009) L954–L964.
[29] M. Faigle, J. Seessle, S. Zug, et al., ATP release from vascular endothelia occurs across Cx43 hemichannels and is attenuated during hypoxia, PLoS ONE 3 (7) (2008) e2801.
[30] S. Godecke, C. Roderigo, C.R. Rose, et al., Thrombin-induced ATP release from human umbilical vein endothelial cells, Am. J. Physiol. 302 (2012) C915–C923.
[31] P. Bodin, G. Burnstock, Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular, J. Cardiovasc. Pharmacol. 38 (2001) 900–908.
[32] M. Neylon, J.M. Marshall, The role of adenosine in the respiratory and cardiovascular response to systemic hypoxia in the rat, J. Physiol. 440 (1991) 529–545.
[33] M.R. Skinner, J.M. Marshall, Studies on the roles of ATP, adenosine and nitric oxide in mediating muscle vasodilatation induced in the rat by acute systemic hypoxia, J. Physiol. 495 (1996) 553–560.
[34] K. Enjyoji, J. Sévigny, Y. Lin, et al., Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation, Nat. Med. 5 (1999) 1010–1017.
[35] F. Kukulski, S.A. Levesque, E.G. Lavoie, et al., Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8, Purinerg Signal 1 (2005) 193–204.
[36] H.K. Eltzschig, J.C. Ibla, G.T. Furuta, et al., Coordinated adenine nucleotide phosphohydrolysis and nucleoside signaling in posthypoxic endothelium: role of ectonucleotidases and adenosine A2B receptors, J. Exp. Med. 198 (2003) 783–796.
[37] X. Li, T. Zhou, X. Zhi, et al., Effect of hypoxia/reoxygenation on CD73 (ecto 5′-nucleotidase) in mouse microvessel endothelial cell lines, Microvasc. Res. 72 (2006) 48–53.