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Chronic hypoxia impairs extracellular nucleotide metabolism and barrier function in pulmonary artery vasa vasorum endothelial cells

Gennady G. Yegutkin,
MediCity Research Laboratory, University of Turku and National Institute of Health and Welfare, Tykistökatu 6A, 20520 Turku, Finland

Mikko Helenius,
MediCity Research Laboratory, University of Turku and National Institute of Health and Welfare, Tykistökatu 6A, 20520 Turku, Finland

Elzbieta Kaczmarek,
Beth Israel Deaconess Medical Center, Harvard Medical School and Center for Vascular Biology Research, Boston, MA, USA

Nana Burns,
Department of Pediatrics, University of Colorado Denver, Aurora, CO 80045, USA

Sirpa Jalkanen,
MediCity Research Laboratory, University of Turku and National Institute of Health and Welfare, Tykistökatu 6A, 20520 Turku, Finland

Kurt Stenmark, and
Department of Pediatrics, University of Colorado Denver, Aurora, CO 80045, USA

Evgenia V. Gerasimovskaya
Department of Pediatrics, University of Colorado Denver, Aurora, CO 80045, USA

Gennady G. Yegutkin: genyeg@utu.fi

Abstract

Vascular remodeling plays a pivotal role in a variety of pathophysiological conditions where hypoxia and inflammation are prominent features. Intravascular ATP, ADP and adenosine are known as important regulators of vascular tone, permeability and homeostasis, however contribution of purinergic signalling to endothelial cell growth and angiogenesis remains poorly understood. By using vasa vasorum endothelial cells (VVEC) isolated from pulmonary artery adventitia of control and chronically hypoxic neonatal calves, these studies were aimed to evaluate the effect of hypoxia on biochemical and functional properties of microvascular endothelial network at the sites of angiogenesis. In comparison with normoxic controls, VVEC from hypoxic animals are characterized by (1) drastically impaired nucleoside triphosphate diphosphohydrolase-1 (NTPDase-1/CD39) and ecto-5′-nucleotidase/CD73 activities with respective increases in basal extracellular ATP and ADP levels (2) higher proliferative responses to low micromolar concentrations of ATP and ADP; and (3) enhanced permeability and disordered adenosinergic control of vascular barrier function (measured as a paracellular flux of 70 kDa fluorescein isothiocyanate-dextran). Together, these results suggest that unique pattern of purine-

Correspondence to: Gennady G. Yegutkin, genyeg@utu.fi.

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mediated angiogenic activation and enhanced leakiness of VVEC from chronically hypoxic vessels may be defined by disordered endothelial nucleotide homeostasis at sites of active neovascularization.

Keywords

Purinergic signaling; Endothelial cells; Vasa vasorum; Hypoxia; NTPDase; Ecto-5′-nucleotidase

Introduction

Pathological vascular remodeling plays a pivotal role in the progression of a variety of diseases and conditions associated with ischemia, hypoxia, and inflammation, such as hypertension, atherosclerosis, restenosis, vascular insufficiency, neoplasia and transplant rejection [1, 2]. The evolving view of abnormal blood vessel growth has directed attention towards the central role of endothelial cells (EC), which undergo a phenotypic switch to an activated endothelial phenotype manifested by the up-regulation of intercellular adhesion molecule (ICAM)-1 expression, and secretion of many pro-angiogenic factors, including vascular endothelial growth factor (VEGF), prostaglandin E_2, interleukin (IL)-1, IL-6, and ATP [2, 3].

Extracellular ATP and other nucleotides (ADP, UTP, UDP) have long been known as regulators of vascular function, particularly involved in control of blood flow, vascular cell proliferation, migration, chemotaxis, and inflammatory responses [4, 5]. In addition, the role of extracellular nucleotides as important regulators of angiogenesis and neovascularization becomes increasingly recognized [6–8]. Evidence is accumulating that vascular EC, as well as smooth muscle, epithelial, hematopoietic and other cells, can release ATP and other agonists in response to sheer stress, hypoxia and other inflammatory and stress-related stimuli. The released nucleotides trigger diverse cell-specific responses through metabotropic (P2Y) and ligand-gated (P2X) receptors [5, 9] followed by their rapid metabolism and interconversion by an array of nucleotide-hydrolyzing and phosphorylating ectoenzymes [10]. Endothelial ecto-nucleoside triphosphate diphosphohy-drolase-1 (NTPDase1/CD39) and ecto-5′-nucleotidase/CD73 are considered the major nucleotide-scavenging enzymes in the vasculature [10–12]. Data on disordered cellular migration, vascular inflammation, enhanced leakiness, pathological angiogenesis and neointima formation in mice deficient in NTPDase1 (*Cd39/Entpd*$^{-/-}$) [13, 14] or ecto-5′-nucleotidase/CD73 [15, 16] further confirmed the importance of coordinated purine homeostasis for proper vascular endothelial functions. Finally, nucleotide-derived adenosine binds to its own receptors that function either by activating (A_{2A} and A_{2B}) or inhibiting (A_1 and A_3) adenylyl cyclase [9, 17] and then either transported into the cell via specific nucleoside transporters or directly metabolized by cell-surface adenosine deaminase [10].

In a neonatal bovine model of arterial pulmonary hypertension, we have shown earlier that marked neo-vascularization of the vasa vasorum occurs in adventitia of chronically hypoxic calves and further demonstrated the importance of extracellular ATP in control of pro-angiogenic responses in vasa vasorum endothelial cell (VVEC) primarily occurring via activation of mitogenic $P2Y_1$ and $P2Y_{13}$ receptors [7, 18, 19]. Elevated levels of extracellular nucleotides are observed under conditions of hypoxia, however it is unknown whether ecto-nucleotidases are altered in EC from vessels undergoing hypoxia-induced remodelling and also whether they influence endothelial phenotype and function. Since a repertoire of purine-converting ectoenzymes modulate local nucleotide concentrations and ultimately, the duration and magnitude of purinergic signaling, here we hypothesized that chronic hypoxia might modulate the pattern of nucleotide turnover thereby shifting EC

towards pro-angiogenic phenotype. The obtained data indicate that the activities of major nucleotide-hydrolyzing enzymes NTPDase1/CD39 and ecto-5′-nucleotidase/CD73 are impaired in VVEC from chronically hypoxic calves, which may serve as important pre-requisite for consistently elevated ATP and ADP levels at sites of vasa vasorum angiogenesis, enhanced EC proliferation and leakiness and eventually, the exacerbation of pathological vascular remodelling.

Materials and methods

Endothelial cells

VVEC were isolated from the adventitial compartments of pulmonary arteries of normoxic (control) and chronically hypoxic (after 2 week exposure to hypobaric hypoxia; P_B = 430 mmHg) male Holstein calves and additionally purified from the co-cultures with adventitial fibroblasts using cloning rings and trypsinization techniques, as described earlier [7]. Isolated VVEC have been shown to express endothelial markers, including vWF, eNOS, and PECAM-1; binding of the lectin *Licopercsicon esculentum*; and incorporate acetylated low density lipoproteins labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate. Cells were grown in high glucose Dulbecco's modified Eagle-Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM L-glutamine and 30 μg/ml Endothelial Cell Growth Supplement (Millipore). Human umbilical vein endothelial cells (HUVEC) were also isolated from fresh umbilical cords and cultured as described earlier [20]. All studies were performed on EC between passages 3 and 6.

Analysis of purine-converting activities

EC were seeded overnight in 96-well flat bottom clear plates, 10,000 cells per well. Enzymatic activities were determined at 37°C in a final volume of 80 μl RPMI-1640 containing 4 mM *β*-glycerophosphate in the following ways: (1) for ATPase and ADPase assays, cultured cells were incubated for 25–30 min with 500 μM [2,8-^{3}H]ATP or [2,8-^{3}H]ADP (Perkin Elmer, Boston, MA) as appropriate substrates; (2) ecto-5′-nucleotidase activity was determined after 45-min incubation with 300 μM [2-^{3}H]AMP (Quotient Bioresearch, GE Healthcare, UK); (2) adenylate kinase and NDP kinase were assayed by incubating the cells with 500 μM [^{3}H]AMP (for 45 min) or [^{3}H]ADP (for 10 min) as respective phosphorus acceptors in the presence of 750 μM *γ*-phosphate-donating ATP. Catalytic reactions were terminated by applying aliquots of the mixture onto Alugram SIL G/UV$_{254}$ sheets (Macherey–Nagel, Duren, Germany). ^{3}H-labeled nucleotides and nucleosides were separated by thin-layer chromatography (TLC) using appropriate solvent mixture and then either quantified by scintillation *β*-counting [21] or exposed to Kodak BioMax MS films for 2 weeks at −70°C and developed by autoradiography. Nucleotide metabolism was also evaluated by incubating the cells for 45 min with 20 μM [*γ*-^{32}P]ATP (Perkin Elmer) followed by TLC separation of the mixture aliquots on Polygram CEL-300 PEI sheets (Macherey–Nagel) with 0.75 M KH_2PO_4 (pH 3.5) as solvent and subsequent autoradiographic analysis.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Cellular mRNA was isolated from 3–4 independent isolations of VVEC from control or hypoxic animals, using RNease spin columms (Qiagen, Valencia, CA, USA). cDNA was synthesized from 1 μg of RNA, using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), according to manufacturer's specifications. Quantitative RT-PCR was performed to measure CD39, CD73 and HIF-1*α* mRNA levels, using gene-specific primers: CD39 (NM_174536)—sense: AATAAAGATGAGCGTCTTAA ACGA; antisense: CCACGGATTTCAATGTCAACGAG; CD73 (NM_174129)—sense:

TCTGAGCGCAAACATTA AAGCC; antisense: CAATCCCCACAACTTCATCACC; HIF-1α (NM_174339)—sense: CTTCGGTATTTAAACC ATTGCAT; antisense: GGACAAACTCCCTAGCCCAA. Reactions were carried out in iTaq Fast SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA, USA) using ABI 7500 Fast Real-time PCR System (Applied Biosystems, Inc., Foster City, CA, USA). The expression of the target genes was normalized to that of the housekeeping gene, β-actin, in each sample.

Western blot analysis

Total cellular extracts were obtained from VVEC cultures grown under standard conditions and separated on 4–12% Bis–Tris polyacrylamide gel (Invitrogen Inc, Carlsbad, CA). PVDF membranes with transferred proteins were blocked in 5% nonfat milk, incubated with a goat polyclonal anti-CD39 antibody (1:500 dilution; Santa Cruz Biotechnology, CA) or with a rabbit monoclonal anti-GAPDH antibody as a loading control (1:1,000; Cell Signaling Technology, Danvers, MA). Membranes were incubated with anti-goat (Santa Cruz Biotechnology, CA) or anti-rabbit (GE Healthcare, UK) HRP-secondary antibody. Immunoreactive bands were visualized using ECL kit (Renaissance, NEN Life Science Product, Boston, MA) and quantified using ImageJ software.

Bioluminescent assay for ATP and ADP measurement

VVEC were seeded onto 24-well tissue culture plate in complete media at the density ~30,000 cells/well. Twenty-four hours later, confluent cells were rinsed and equilibrated at 37°C in the starting volume of 1 ml Krebs–Ringer phosphate glucose (KRPG; comprising: 145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, and 5.5 mM glucose; pH 7.35). Aliquots of the medium (100 μl) were collected after 1 and 2.5 h of incubation, clarified by centrifugation, and assayed for ATP and ADP concentrations using ATPlite assay kit with a long-lived luminescent signal (Perkin Elmer, Groningen, The Netherlands) under the conditions delineated experimentally. Specifically, 40-μl aliquots were transferred into two parallel wells of white non-phosphorescent 96-well microplate containing 100 μl distilled H_2O with (A) or without (B) mixture of 200 μM UTP and 5 U/ml of NDP kinase from baker's yeast, S. cerevisiae (Sigma). Subsequent to addition of 50 μl ATP-monitoring reagent, luminescence of the samples was measured using Tecan Infinite-M200 multifunctional microplate reader (Salzburg, Austria). The differences in luminescence signals between well "A" (ATP + ADP) and "B" (only ATP) allowed quantifying the concentration of ADP, which was converted into ATP through the NDP kinase-mediated reaction in the presence of exogenous UTP. Such approach allows simultaneous measurement of ATP and ADP content within the same sample.

Proliferation assay

Cells were plated in 24-well plates at a density of 1.2×10^4 cells per well in DMEM supplemented with 10% FBS. On the next day cells were rinsed with phosphate-buffered saline (PBS), and incubated in DMEM without serum for 72 h. Cells were stimulated with exogenous ATP, ADP or adenosine (10^{-9}–10^{-3} M) in the presence of 0.125 μCi of [*methyl*-^{3}H]-thymidine (NEN Life Science Products, Boston, MA) for 24 h. Incorporated radioactivity was determined as previously described [7].

Transendothelial permeability assays

Permeability assays were performed using HTS FluoroBlok™ 96-Multiwell Insert system with 8 μm pore size (BD Falcon) and FITC-conjugated dextran 70 kDa (Molecular Probes®, Invitrogen). VVEC were seeded onto permeable fibronectin-coated inserts and cultured for 24–48 h in complete medium. The integrity of the cells was ascertained by inability of human red blood cells to pass the inserts after their co-incubation with endothelial

monolayer for 4 h at 37°C. Confluent VVEC were then transferred to another 96-square-well flat bottom plate. ATP, AMP and adenosine diluted in DMEM, as well as DMSO solutions of 5′-*N*-ethyl-carboxamide-adenosine (NECA) and 5′-(N-cyclopropyl)-carboxamido-adenosine (CPCA) (all from Sigma) were added at final concentrations 10 μM to both the upper and lower chambers containing 50 and 150 μl of serum-free medium, respectively. Vehicle-treated cells contained DMEM with or without 0.05% DMSO. After 30-min pre-incubation at 37°C, 10 μl of 35 μM FITC-dextran was applied onto the cells and its flux was monitored by periodical measurement of fluorescence (excitation 485 nm; emission 545 nm) in the lower chambers, using Tecan Infinite M200. According to the cell viability test with trypan blue staining, none of the agonists used had a toxic effect on VVEC.

cAMP assay

Cell-derived cAMP was assayed using LANCE® cAMP Detection Kit (PerkinElmer), which combines homogenous Time-Resolved FRET technology with red-shifted Fluor® Dye chemistry. Briefly, detached VVEC (~20,000 cells) were incubated in Dulbecco's phosphate buffered saline containing 0.1% BSA and 0.5 mM isobutyl-methyl-xanthine (IBMX, Sigma). After addition of Alexa Fluor® 647 anti-cAMP antibody, adenylyl cyclase activity was triggered by stimulating the cells for 45 min with various concentrations of G-protein-coupled receptor agonists forskolin, NECA, CPCA and adenosine (10^{-7}–10^{-4} M; all from Sigma). After addition of appropriate "detection mix" (containing Eu-labelled streptavidin and biotinylated cAMP), FRET emission of the obtained cAMP complex was monitored at excitation and emission wavelengths 340 and 665 nm, respectively (Tecan Infinite M200). For quenching corrections, residual fluorescence emission of the samples was additionally recorded at 615 nm. Calibration curves were generated for each experiment using cAMP standard serial dilutions.

Statistical analyses

Parallel assays were performed in duplicates with at least two separate VVEC isolates obtained from different hypoxic and normoxic animals. Most data are presented as column bars (mean ± SEM). Kinetic data from cAMP and permeability studies were subjected to appropriate nonlinear regression analyses using GraphPad Prism™ 4.03 software (San Diego, CA). Data sets were examined by analysis of variance (ANOVA) and significance level was set at $P < 0.05$.

Results

Evidence for co-existence of ATP-consuming and ATP-generating endothelial pathways and impaired nucleotide catabolism in VVEC from hypoxic animals

Autoradiographic TLC analysis of endothelial nucleotide-converting pathways was performed using tracer nucleotide substrates and cultured VVEC as enzyme source. As shown in Fig. 1a, incubation of VVEC isolated from control calves with 20 μM [γ-^{32}P]ATP caused its conversion into ADP and $^{32}P_i$ without detectable formation of $^{32}PP_i$ pyrophosphate (lane 2). Joint addition of [γ-^{32}P]ATP and unlabeled AMP (lane 3) or UDP (lane 5) induced respective formation of [^{32}P]ADP or [^{32}P]UTP, with the former reaction being prevented by specific adenylate kinase inhibitor diadenosine pentaphosphate (Ap_5A; lane 4). VVEC incubation with another tracer nucleotide [^{3}H]AMP (50 μM) revealed its dephosphorylation into [^{3}H]adenosine (Fig. 1b, lane 1), and this catalytic reaction was inhibited in the presence of specific ecto-5′-nucleotidase inhibitor α,β-methylene ADP (APCP; lane 2). Exogenous ATP also prevented the hydrolysis of [^{3}H]AMP and concurrently triggered its sequential transphosphorylation via [^{3}H]ADP into [^{3}H]ATP (lane 3), which was prevented by Ap_5A (lane 4).

In comparison with normoxic cells (Fig. 1c, lane 2), VVEC from hypoxic animals possessed markedly diminished [^{3}H]AMP-hydrolyzing capability (lane 1). Incubation of VVEC from control animals with [^{3}H]ADP as an initial substrate also revealed its sequential breakdown via [^{3}H]AMP into [^{3}H]adenosine (panel C, lane 5). By contrast, hypoxic VVEC exhibited relatively low [^{3}H]ADP-hydrolyzing activity with [^{3}H]AMP being a major end-reaction product and at the same time, triggered slight backward conversion of [^{3}H]ADP into [^{3}H]ATP occurring even in the absence of exogenous γ-phosphate-donating ATP (lane 4). Pre-treatment of control VVEC with increasing concentrations of known NTPDase1 inhibitor sodium polyoxotungstate-1 (POM-1) [22] also triggered progressive inhibition of ADPase activity with IC_{50} value of ~1.5 μM (Fig. 1d). These autoradiographic studies corroborate our previous findings showing the presence of various purine-converting activities in EC [20, 23] that involves the enzymes of inactivating cascade NTPDase1/CD39 and ecto-5′-nucleotidase/CD73 and the counteracting phosphorylating enzymes comprising adenylate kinase and NDP kinase. Moreover, these studies suggest the inhibitory effect of chronic hypoxia on endothelial nucleotide-hydrolyzing enzymes.

Hypoxic VVEC display diminished NTPDase and ecto-5′-nucleotidase activities and maintain elevated extracellular ATP and ADP levels

Specific ecto-nucleotidase activities were then assayed using saturating concentrations of ^{3}H-labelled ATP, ADP and AMP. Consistent with above autoradiographic data, the rates of [^{3}H]ADP and especially [^{3}H]AMP hydrolyses were significantly diminished in VVEC from hypoxic calves, as compared with normoxic controls (Fig. 2a). Concurrent measurement of counteracting ecto-adenylate kinase and NDP kinase activities did not reveal any differences in ATP-mediated [^{3}H]AMP and [^{3}H]ADP phosphorylation between control and hypoxic VVEC (Fig. 2a). Along with evidence for an inability of chronic hypoxia to affect the backward ATP-generating pathway, such unchanged kinase activities may additionally serve as appropriate “internal control” for comparable assay conditions among the normoxic and hypoxic EC.

Figure 2b represents a schematic view of relative rates of ^{3}H-nucleotide hydrolyses by bovine EC. Compared with normoxic controls, all ecto-nucleotidase activities were decreased in VVEC from hypoxic animals. For additional comparative analysis of species variations, we also included HUVEC in this study. Interestingly, both control VVEC and HUVEC utilized ADP as preferred substrate with ADP:ATP hydrolysis ratio of ~1.4, whereas hypoxic VVEC exhibited equal specificity for both ATP and ADP substrates, mainly due to the relatively low ADP-scavenging capability of NTPDase. It may be anticipated that diminished ecto-nucleotidase activities would lead to local accumulation of extracellular nucleotides (ATP and ADP) in the cell vicinity. To test this hypothesis, we devised a sensitive enzyme-coupled assay, in which ADP reacts with exogenously applied UTP in the presence of NDP kinase to generate UDP and ATP, with the latter nucleotide being measured by a coupled luciferin/luciferase reaction. As shown in Fig. 2c, conditioned medium from control non-stimulated VVEC contains low nanomolar concentrations of ATP and ADP, whereas basal extracellular levels of both nucleotides were ~3-times higher in VVEC isolated from hypoxic animals, even after 2.5-h equilibration.

Hypoxia-induced decrease in endothelial ecto-nucleotidase activities is not accompanied by changes at the RNA or protein levels

Next, we evaluated whether the above decreases in endothelial ecto-nucleotidase activities might be related to hypoxia-induced changes in gene expression and/or protein level. Furthermore, we analyzed the expression of known hypoxia-inducible transcription factor HIF-1α, which may regulate the expression of CD73 in endothelial and other cells under various hypoxic and ischemic conditions [24]. Real time PCR analysis did not reveal a

significant differences in CD39 (NTPDase1) mRNA between VVEC from control and hypoxic animals. Rather, there was a trend of concurrently increased CD73 (ecto-5′-nucleotidase) and HIF-1α mRNA levels in VVEC from hypoxic calves, although it did not reach statistical significance (Fig. 3a). Additional Western blot analysis of VVEC lysates using anti-CD39 antibody also demonstrated that chronic hypoxia does not affect total expression level of CD39/NTPDase1 (Fig. 3b). Unfortunately, the available anti-CD73 antibodies which were successfully employed earlier for Western blot and immunofluorescence staining in HUVEC [23], did not generate any detectable signal in cultured VVEC. Most likely, this reflects the inability of these antibodies generated against human CD73 to recognize bovine protein and/or the presence of relatively low ecto-5′-nucleotidase activities in VVEC from control and especially hypoxic animals, as compared with HUVEC (see Fig. 2b).

Effect of exogenous purines on angiogenic properties of cultured VVEC

We then questioned whether changes in nucleotide homeostasis affect the proliferative responses of EC, thus contributing to the angiogenic expansion of the vasa vasorum. For this purpose, VVEC were cultured in the absence of serum for 72 h followed by additional 24-h treatment with increasing concentrations of ATP, ADP or adenosine in the presence of [^{3}H]-thymidine. Noteworthy, long-term serum starvation and challenging to various purinergic agonists was not accompanied by any signs of apoptosis, as ascertained by Annexin V binding to VVEC [18]. Exogenous ATP and ADP and, to a lesser extent, adenosine stimulated gradual increases in DNA synthesis in VVEC studied (Fig. 4). Interestingly, both ATP and ADP increased [^{3}H]thymidine incorporation in VVEC from hypoxic calves at the concentration range of 10^{-6}–10^{-3} M, whereas significant proliferative responses in control cells were observed only at higher concentrations of ATP (10^{-5}–10^{-3} M) and ADP (10^{-4}–10^{-3} M). These data suggest the enhanced proliferative capability of former cells in response to low micromolar nucleotide levels.

Adenosinergic signalling controls vascular barrier function in VVEC isolated from control but not chronically hypoxic calves

For further elucidation of purinergic mechanisms underlying the well-established hypoxia-induced increase in lung vascular permeability [16, 25, 26], permeability assay was performed by measuring flux of FITC-dextran across EC monolayers grown on permeable inserts. Kinetic analysis of the obtained saturated curves confirmed the ability of VVEC isolated from control animals to regulate paracellular flux of macromolecules. This barrier function can be further enhanced after cell pre-treatment with non-selective (NECA) or A_2-selective (CPCA) agonist of adenosine receptors (Fig. 5). Likewise, adenosine precursors ATP and AMP, but not adenosine itself, significantly decreased the flux of FITC-dextran through control VVEC, although not as potently as CPCA and NECA. In striking contrast, VVEC from hypoxic animals were characterized by enhanced leakiness, manifested in shorter time required for flux of 50% of maximal fluorescence (~15 min in hypoxic vs. 40 min in normoxic cells), without any differences in B_{max} values. Furthermore, no changes in FITC-dextran clearance were seen in studies with hypoxic calves-derived VVEC pre-treated with various purinergic agents (Fig. 5b).

We also measured the capacity of VVEC to generate cAMP, known to be involved in control of vascular barrier function [27]. Adenylyl cyclase was assayed using Perkin Elmer LANCE® kit, which allows detecting cAMP at low sub-nanomolar levels (Fig. 6a, Inset). VVEC incubation with classical adenylyl cyclase activator, forskolin, caused concentration-dependent increase in cAMP levels (Fig. 6a). Subsequent nonlinear analysis of the obtained sigmoidal dose–response curves did not reveal significant differences in the measured kinetic parameters between control (B_{max} = 518 ± 133 nM, EC_{50} = 1.51 μM) and hypoxic

(B_{max} = 393 ± 52 nM, EC_{50} = 1.57 μM) animals. In comparison with forskolin, CPCA and NECA served as less potent activators of adenylyl cyclase. Nevertheless, comparable magnitudes of cAMP responses were observed after incubation of VVEC from control and hypoxic animals with micromolar concentrations of these adenosine receptor agonists (Fig. 6b). Interestingly, adenosine itself did not increase cAMP level even at high (100 μM) concentration (Fig. 6b). This apparent discrepancy may be explained by co-expression of cAMP activating (A_{2a}) and inhibiting (A_1 and A_3) adenosine receptors on studied VVEC [19], partial desensitization of A_2 receptors by endogenous adenosine [28] and/or rapid metabolism and uptake of the added nucleoside by the cell [10]. These data suggest that enhanced leakiness and impaired purinergic control of barrier function in VVEC from hypoxic calves presumably are not defined by defective downstream cAMP-mediated signalling pathways.

Discussion

In this study we sought to further elucidate cellular mechanisms contributing to the previously described expansion of vasa vasorum network in the pulmonary arteries from chronically hypoxic neonatal calves [2, 7, 29], with particular emphasis on the regulatory role of endothelial purine-converting ectoenzymes in hypoxia-induced EC leakiness and neovascularization. Salient findings are that, in comparison with normoxic controls, VVEC from hypoxic animals are characterized by: (1) diminished NTPDase and ecto-5′-nucleotidase activities with respective increase in basal extracellular ATP and ADP levels; (2) higher proliferative responses to low micromolar concentrations of ATP and ADP; and (3) enhanced paracellular permeability and disordered purinergic control of vascular barrier function.

By comparing the pattern of nucleotide metabolism in VVEC isolated from control and hypoxic animals, here we showed that prolonged hypoxic exposure is accompanied by marked decreases in NTPDase1/CD39 and ecto-5′-nucleotidase/CD73 activities without significant changes in the counteracting adenylate kinase and NDP kinase activities. These data are basically consistent with current concepts proposing that vascular endothelial NTPDase1/CD39 can be diminished and even lost under various conditions of inflammatory or oxidant stress, including ischemia–reperfusion injury, transplantation and rejection processes [1, 30]. Previous in vitro studies also demonstrated that long-term hypoxic exposure of adventitial EC and fibroblasts (3% for ≥14 days) was accompanied by decrease in cell-surface ecto-ATPase activity [31]. On the other hand, acute hypoxia may trigger substantial up-regulation of both NTPDase1/CD39 and ecto-5′-nucleotidase/CD73 activities in post-hypoxic endothelial and epithelial cells [32]. Significant up-regulation of endothelial nucleotidase activities had also been reported in murine models of acute hypoxia, which in turn affected vascular leakage in a vascular bed-specific manner [26]. Such apparent discrepancy may be explained by different mechanisms underlying the acute and chronic effects of hypoxia. In addition, cellular composition of the proximal pulmonary and systemic vessels of larger mammalian species (including the cow and human) exhibit tremendous functional and morphological heterogeneity and their responses to hypoxia are more complex that that of the rodent species [2]. Other components of purinergic signaling cascade can also be substantially modulated during acute or chronic exposure of endothelial and other cells to acute or chronic hypoxia, including A_{2B} adenosine receptors, adenosine deaminase, and adenosine transporters [33, 34].

The expression of ecto-5′-nucleotidase/CD73 is closely associated with HIF-1α, with both transcripts known to be co-ordinately up-regulated under various hypoxic and ischemic conditions [24, 35]. While clear trend of concurrent hypoxia-mediated increases of CD73 and HIF-1α mRNA levels was also observed in our study, this slight transcriptional

induction is assisted with an opposing decrease of ecto-5′-nucleotidase catalytic activity in VVEC from hypoxic animals. Probably, the diminished activity of this glycosyl-phosphotidylinositol anchored enzyme is defined by hypoxia-induced post-translational changes in the enzyme expression, which may be particularly down-regulated during enzyme inhibition by precursor nucleotides ATP and ADP [20] or circulating leukocytes [23], as well as due to insufficient formation of adenosine, which generally provides a positive loop for regulated expression of endothelial CD73 [36]. Regarding another nucleotide-hydrolyzing enzyme NTPDase1/CD39, it is pertinent to mention that cell-surface NTPDases exist either in monomeric or in higher homooligomeric (dimeric to tetrameric) states and their activities may be specifically controlled by oligomerization state [12, 37, 38]. For instance, some lectins and antibodies would stabilize the enzyme oligomers with consequent stimulation of ecto-ATPase activity, whereas various agents and conditions increasing membrane fluidity and weakening the interaction between monomers (suramin, certain detergents) inhibit the ecto-ATPase activity [37]. Other factors potentially involved in the regulation of cell surface NTPDase may include oxidative cross-linking of cysteine residues in the enzyme transmembrane domains with respective reduction of their rotational mobility and marked loss of catalytic activity [38].

By devising a novel enzyme-coupled ATP- and ADP-sensing bioluminescent assay, we have also found that in comparison with control cells, VVEC from hypoxic animals constitutively maintain ~3-times higher levels of both nucleotides. While at first sight the revealed hypoxia-induced increases of nanomolar ATP and ADP levels may look insignificant in terms of their physiological pertinence, it should be kept in mind that measured extracellular nucleotide concentrations are often underestimated due to their rapid enzymatic hydrolysis [28] and in addition, relatively high (micromolar) ATP concentrations can be spatially confined in the pericellular space with only minor nucleotide portion being convected into the bulk medium [10]. The latter suggestion is supported by our autoradiographic data showing the ability of hypoxic VVEC to slightly convert [^{3}H]ADP into [^{3}H]ATP even in the absence of exogenous γ-phosphate-donating ATP (see Fig. 1c, lane 4). Interestingly, similar “autophosphorylation” pattern has been reported earlier for human leukemic cell lines [39]. These findings, in concert with data on markedly diminished ecto-nucleotidase activities, provide evidence that VVEC from hypoxic animals maintain constitutively elevated pericellular ATP and ADP levels at concentrations sufficient for activation of P2 receptors. In turn, these shifts in endothelial nucleotide homeostasis may serve as important determinant for development of unique pro-angiogenic phenotype. This suggestion is further supported by our data on ^{3}H-thymidine incorporation showing that in comparison with control cells, lower threshold ATP and ADP concentrations (1 μM) are required for triggering proliferative responses in VVEC from hypoxic animals. Noteworthy, other EC derived from the luminal surface of large pulmonary or systemic vessels possessed very little proliferative capacity in response to ATP [7, 29] and at the same time displayed 2 to 3-times higher NTPDase activities than VVEC [29].

Another important consequence of hypoxia-induced changes in nucleotide turnover concerns endothelial barrier function, which may be regulated either via direct ATP activation of P2Y-mediated protein kinase A pathways [40] or, more commonly, through ectoenzymatic conversion of nucleotides to adenosine and subsequent activation of A_{2B} receptors [23, 32, 41, 42]. Consistent with previous data showing that hypoxic exposure increases lung vascular permeability measured by direct protein extravasation [2], our paracellular permeability assays also revealed the enhanced leakiness of VVEC from hypoxic calves. Strikingly, adenine nucleotides (ATP, AMP) and adenosine receptor agonists were able to decrease the flux of FITC-dextran through VVEC monolayers obtained from control, but not hypoxic animals. Since pulmonary endothelial barrier is dynamically regulated by cAMP-mediated signaling cascades, any perturbations in production or compartmentalization of

this second messenger would potentially lead to disturbances in barrier integrity and abnormal hyperpermeability [27]. However, no significant differences in cAMP-mediated signaling pathways were detected in our study with VVEC isolated from control and hypoxic animals. Most likely, the increased leakiness of the latter EC and inability of purinergic agents to regulate their barrier function might be due to the concurrently down-regulated ecto-5′-nucleotidase/CD73 activity and respective disturbance of extracellular adenosine homeostasis. Such possibility is indirectly supported by previous data on dramatically increased vascular leakage in lungs from $CD73^{-/-}$ mice [16], as well as by in vitro permeability studies showing the key regulatory role of ecto-5′-nucleotidase/CD73 in control of endothelial barrier function and transendothelial leukocyte migration [23, 32, 36, 41].

Collectively, data from this study suggest that down-regulation of endothelial ecto-nucleotidases and accumulation of extracellular ATP and ADP might provide the important autocrine loop for enhanced cell proliferation, leakiness and pathological vascular remodelling during prolonged hypoxic exposure. These findings are consistent with a current view that extracellular purines play an important role in angiogenesis and provide a novel evidence of the key regulatory function of purinergic ecto-enzymes in the hypoxia-induced neovascularization.

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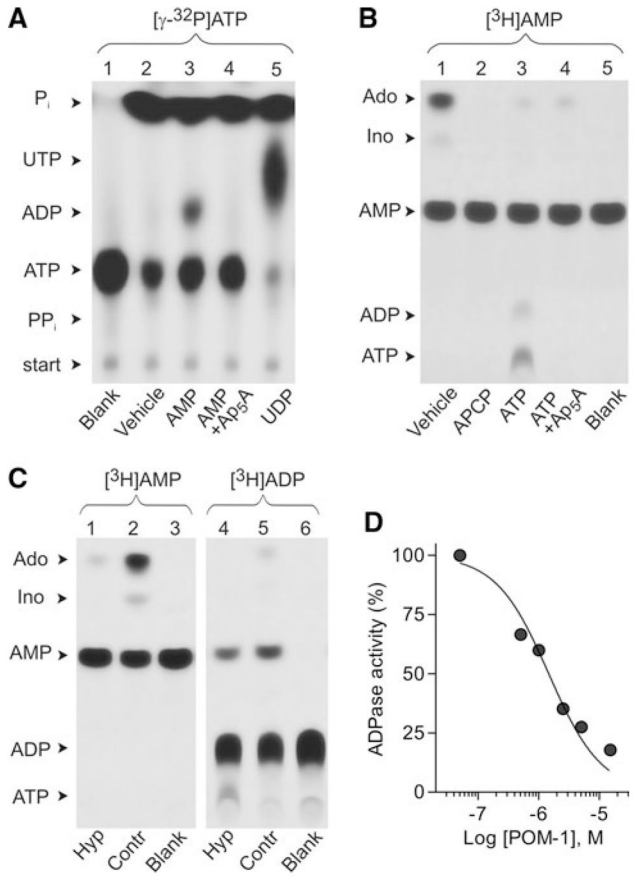

Fig. 1.

TLC analysis of endothelial purine-converting pathways. VVEC from control calves were incubated with 20 μM [γ-^{32}P]ATP (**a**) or 50 μM [^{3}H]AMP (**b**) in the absence (*vehicle*) and presence of 400 μM AMP, UDP and ATP, as indicated. Some cells were also pre-treated with 50 μM Ap_5A or APCP prior to addition of tracer substrates. **c** Comparative analysis of nucleotide metabolism in VVEC from control (*contr*) and hypoxic (*hyp*) animals was also performed using 50 μM [^{3}H]AMP and 100 μM [^{3}H]AMP as initial substrates. Aliquots of the reaction mixture were separated by TLC and developed by autoradiography. The *blanks* show the radiochemical purities of ^{32}P- and ^{3}H-labeled nucleotides in the absence of EC. *Arrows* indicate the positions of nucleotide standards, inorganic phosphate (P_i) and pyrophosphate (PP_i). **d** Control VVEC were pre-treated for 30 min with increasing concentrations of NTPDase1 inhibitor POM-1 prior to the addition of 400 μM [^{3}H]ADP. The results are plotted as the percentage of maximal [^{3}H]ADP hydrolysis determined in the absence of inhibitor

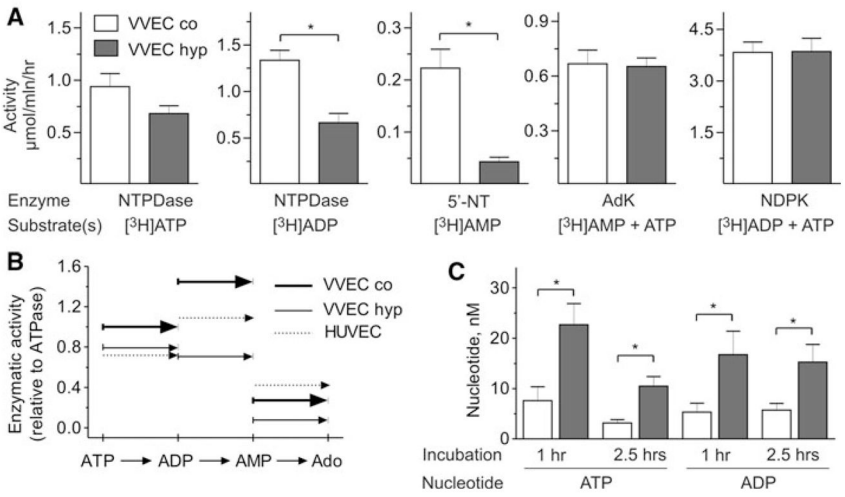

Fig. 2.

Impaired nucleotide catabolism and elevated extracellular ATP and ADP levels in VVEC from hypoxic animals. **a** Specific NTPDase (ATPase/ADPase), ecto-5′-nucleotidase (*5′-NT*), adenylate kinase (*AdK*) and NDP kinase (*NDPK*) activities were determined in VVEC from control (*Co*) and hypoxic (*Hyp*) calves by TLC using saturating concentrations of indicated ^{3}H-labeled and unlabeled nucleotides. Activities are expressed as nanomoles of ^{3}H-substrate metabolized by 10^{6} cells per hour and represented as mean ± SEM for at least 10 independent assays performed with VVEC isolates from three different animals. **b** Comparative analysis of ecto-nucleotidase activities in various endothelial cells. The *arrows* show averaged activities from 8 to 10 different measurements performed in VVEC from control (*bold line*), and hypoxic (*thin line*) calves, as well as from cultured HUVEC, as an additional control (*doted line*). All activities are normalized relative to the ATPase in control VVEC (defined as 1). **c** Conditioned medium was periodically collected from cultured VVEC and assayed for extracellular ATP and ADP using enzyme-coupled luciferin-luciferase technique (mean ± SEM, $n = 6–8$). *$P < 0.05$ as compared with VVEC from control animals

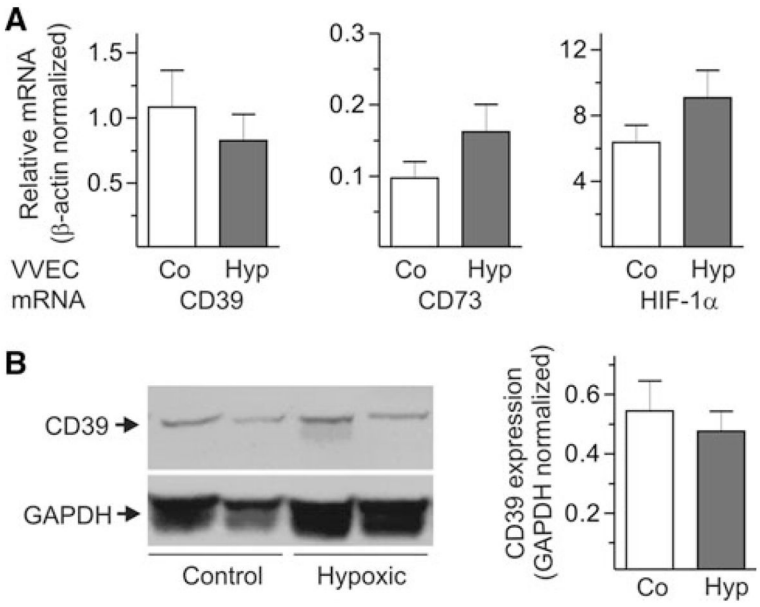

Fig. 3.

Chronic hypoxia does not significantly change the expression levels of CD39 and CD73 in VVEC. **a** Analysis of CD39, CD73 and HIF-1α mRNA levels in VVEC from control and hypoxic animals by qPCR. Data were normalized versus β-actin for each gene. **b** Western blot analysis of CD39 expression was performed in total VVEC lysates by using anti-CD39 antibody and anti-GAPDH as a loading control. Representative images of CD39 and GAPDH immunoreactive bands in VVEC isolated from two different control and hypoxic animals are shown on the *left panel*. The *bars* represent quantitative analysis of relative CD39 levels (normalized to GAPDH; mean ± SEM) determined in VVEC from control (n = 5) and hypoxic (n = 7) calves

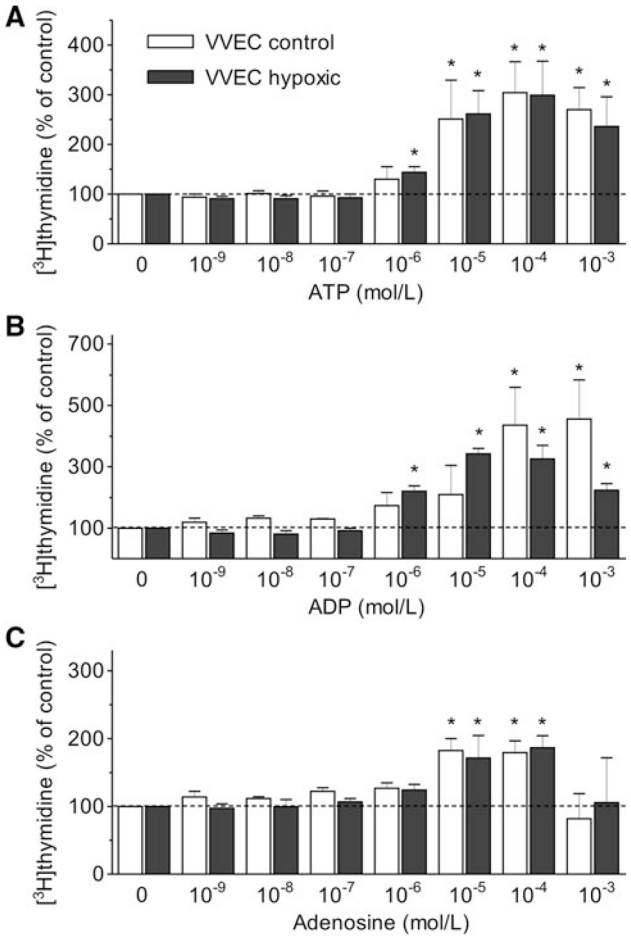

Fig. 4.

Extracellular purines exert different proliferative responses in VVEC from control and hypoxic animals. Growth arrested VVEC were incubated for 24 h with 0.125 μCi [^{3}H]thymidine in the absence and presence of various concentrations (10^{-9}–10^{-3} M) of ATP (**a**), ADP (**b**) and adenosine (**c**), as indicated. The results are expressed as percentage of basal [^{3}H]thymidine incorporation determined in the absence of exogenously applied purines (mean ± SEM; $n \geq 3$). *$P < 0.05$ as compared with non-stimulated cells

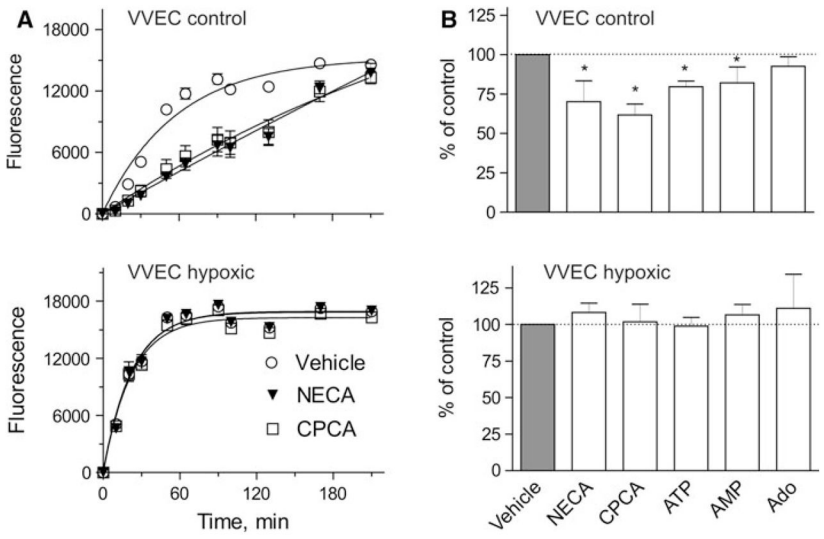

Fig. 5.

Role of adenosinergic signaling in endothelial barrier function. VVEC monolayers from control and hypoxic calves grown on permeable inserts were pre-treated for 30 min without or with various purinergic agonists (10 μM each), as indicated. **a** After applying FITC-dextran onto the cells, the time course of its paracellular flux was determined by periodical measurement of fluorescence intensity in the lower chamber. **b** The *bars* show the amount of FITC-dextran passed across the treated cells after 60 min, expressed as percentage of fluorescence in control vehicle-treated wells (mean ± SEM, $n = 4$). *$P < 0.05$ as compared with vehicle

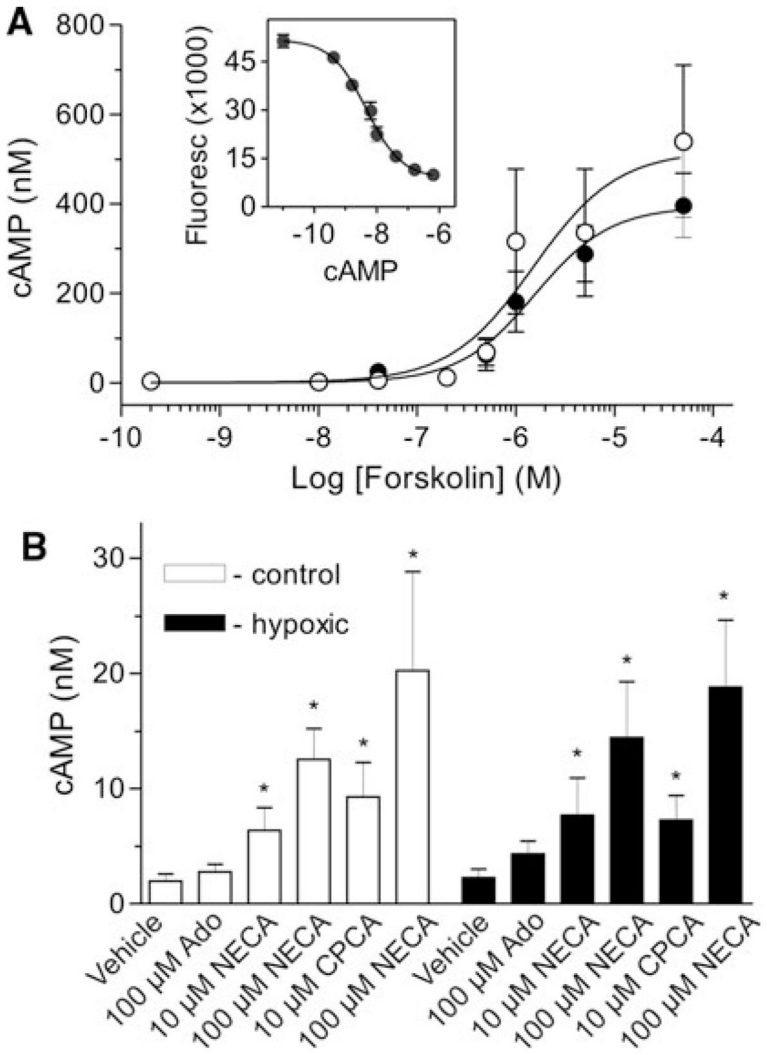

Fig. 6.

Effect of various agonists on adenylyl cyclase activity in VVEC from control and hypoxic calves. VVEC were incubated with various concentrations of forskolin (**a**), as well as adenosine and adenosine receptor agonists, NECA or CPCA (**b**). Adenylyl cyclase activity was measured using a cAMP-detecting LANCE® kit. The obtained raw fluorescence values were transformed and interpolated into cAMP concentration based on standard cAMP calibration curve generated for each experiment (*inset* in Panel **a**). The *graphs* show cAMP concentration from at least three independent experiments (mean ± SEM). **P* < 0.05 as compared with basal cAMP measured in vehicle-treated cells