RESEARCH ARTICLE

Glycolysis and oxidative phosphorylation are essential for purinergic receptor-mediated angiogenic responses in vasa vasorum endothelial cells

Martin Lapel, Philip Weston, Derek Strassheim, Vijaya Karoor, Nana Burns, Taras Lyubchenko, Petr Paucek, Kurt R. Stenmark, and Evgenia V. Gerasimovskaya

1Department of Pediatrics, University of Colorado Denver, Aurora, Colorado; 2Department of Medicine, University of Colorado Denver, Aurora, Colorado; and 3Department of Pharmacology, University of Colorado Denver, Aurora, Colorado

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STUDIES FROM SEVERAL MODELS SUGGEST THAT DIFFERENTIATED EC REPRESENT A MOSTLY GLYCOLYTIC PHENOTYPE WITH RELATIVELY FEW MITOCHONDRIA, DENOTING OXIDATIVE PHOSPHORYLATION (OXPHOS) AS THE NONPRIMARY SOURCE OF ATP GENERATION IN EC (27, 76). IT HAS BEEN DEMONSTRATED THAT, SIMILAR TO HIGHLY PROLIFERATIVE CANCER CELLS, EC INCREASE THEIR GLYCOLYTIC FLUX IN RESPONSE TO ANGIOGENIC ACTIVATION (36, 72). THE INDUCTION OF AEROBIC GLYCOLYSIS (WARBURG EFFECT) WAS SHOWN IN EC PROLIFERATING UNDER HYPOXIC CONDITIONS, THEREBY DIRECTING CELLULAR ENERGY METABOLISM TO VASCULAR REMODELING (26, 51, 72, 82). RELIANCE ON AEROBIC GLYCOLYSIS WAS OBSERVED IN PROLIFERATIVE PA LUNG MICROVASCULAR EC (52) AND IN TIP EC UNDERGOING FLOPIDIA FORMATION DURING VESSEL SPROUTING (22, 23). IT HAS ALSO BEEN REPORTED THAT PA EC FROM PATIENTS WITH IPH EXHIBIT AN ABNORMAL METABOLIC PHENOTYPE, CHARACTERIZED BY A METABOLIC SWITCH TO GLYCOLYSIS ACCOMPANYING REDUCED NUMBERS OF MITOCHONDRIA (82).

MEANWHILE, INCREASING DATA DEMONSTRATE THAT SOME HIGHLY PROLIFERATIVE TUMOR CELLS EXHIBIT GREAT DIVERSITY OF ENERGY UTILIZATION IN GROWTH RESPONSES AND MAY RETAIN THE OXPHOS METABOLISM DURING ONCOGENIC TRANSFORMATION (40, 65). IN ADDITION, CARDIOMYOCYTES, MESENCHYMAL STEM CELLS, AND INFAMMATORY AND TUMOR STEMS ARE BIVALENT IN THEIR ENERGY PRODUCTION AND MAY UNDERGO A SWITCH FROM GLYCOLYSIS TO MITOCHONDRIAL RESPIRATION DURING DIFFERENTIATION (53, 56, 62, 68). EVEN THOUGH EC ARE CONSIDERED A “GLYCOLYTIC” CELL TYPE, RECENT DATA ALSO SUGGEST THAT MITOCHONDRIAL FUNCTION IS IMPORTANT IN HOMEOSTATIC REGULATION AND ANGIOGENIC CAPACITY OF ENDOTHELIUM (20, 25, 64). PERINUCLEAR CLUSTERING OF MITOCHONDRIA IN LUNG EC HAS BEEN DEMONSTRATED TO CONTRIBUTE TO ROS-DEPENDENT VEGF PRODUCTION (5). THERE IS ALSO EVIDENCE THAT PROLIFERATING EC INCREASEDLY DEPEND ON MITOCHONDRIAL OXPHOS AND, IN PARTICULAR, ON THE MITOCHONDRIAL PROTON GRADIENT (18). TOGETHER, THESE OBSERVATIONS SUPPORT AN ONGOING METABOLIC REPROGRAMMING OF ENDOTHELIAL CELLS AND TUMOR STEM CELLS DURING ANGIOGENIC ACTIVATION (36, 72).

ADDRESS FOR REPRINT REQUESTS AND OTHER CORRESPONDENCE: E. Gerasimovskaya, Univ. of Colorado Denver, Pediatric Critical Care Medicine, Box B131, 12700 E. 19th Ave., Research 2, Rm. 612, Aurora, CO 80045 (e-mail: evgenia.gerasimovskaya@ucdenver.edu).
NURSING AND METABOLIC REGULATION OF VASA VASORUM EC

vations suggest the possibility that, depending on the activation and/or differentiation status, EC can undergo phenotypic and metabolic reprogramming. The relative contribution of endothelial glycolysis and OXPHOS to angiogenesis remains under investigation.

Regulation of angiogenesis via receptor-mediated signaling pathways has been fairly well studied. In contrast, the control of metabolism by receptor-mediated signaling has not, although this concept has been represented in several reports (28, 44, 72). Previously, our laboratory demonstrated that ATP can be released by VVEC in response to hypoxia and play a critical role in an autocrine/paracrine regulation of hypoxia-induced VV angiogenesis (80). However, regulation of cellular energy pathways via purinergic receptor-mediated signaling and whether it results in EC metabolic reprogramming remains to be determined. In our present study, we used VVEC isolated from the vascularized areas of PA adventitia of chronically hypoxic calves that represent disease-relevant cell model to investigate signaling and metabolic responses involved in angiogenesis. Our data show that glycolysis and OXPHOS are both essential for VVEC angiogenesis and demonstrate a distinct role of P2 purinergic receptor (P2R) and P1 purinergic receptor (P1R) agonists in stimulating VVEC angiogenic responses, glycolysis, and OXPHOS. We also demonstrate a role of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in the regulation of several glycolytic and OXPHOS enzymes, as well as the facilitative glucose transporter GLUT-1. Based on results from our study, we speculate that regulation of VV angiogenesis by metabolic pathways and purinergic receptor agonists may contribute to pathological vascular remodeling in PH.

METHODS

**VVEC culture.** VVEC were isolated from vascularized areas of PA adventitia of chronically hypoxic (14 days exposed to hypobaric hypoxia; barometric pressure = 430 mmHg) male Holstein calves, as previously described (30). Standard veterinary care was used following institutional guidelines, and the procedure was approved by the Institutional Animal Care and Use Committee (Department of Physiology, and phenotypic characteristics.

**DNA synthesis and proliferation assays.** To determine the rate of DNA synthesis, VVEC were plated in 24-well plates at a density of 1.2 × 10^3 cells/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 preincubated with 2-deoxyglucose (2-DG; 2 mM), oligomycin (100 ng/ml), rotenone (0.1 μM), or FCCP (5 μM) for 20 min and stimulated with ATP, 2-methylthioadenosine diphosphate trisodium salt (MeSADP), or adenosine (100 μM each) in the presence of 0.125 μCi of [methyl-^3H]thymidine (NEN Life Science Products, Boston, MA) for 24 h. Incorporated radioactivity was measured using a β-counter, as previously described (30). To determine the effects of metabolic inhibitors on VVEC proliferation, cells were plated in 96-well plates at a density of 1.2 × 10^3 cells/well in DMEM supplemented with 1% FBS and grew in the presence of indicated metabolic inhibitors. Incubation media with all indicated components were changed each second day, and cell proliferation rate was assessed using CyQuant proliferation kit (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Fluorescence intensity was determined using a plate reader (BMG Labtech).

**Cell migration.** To assess the effects of metabolic inhibitors on angiogenic activity of VVEC, migration and tube formation in vitro assays were performed as previously described (30). Growth arrested cells (DMEM without serum, 72 h) were seeded on top of the transwell-permeable support of the Boyden chamber (8-mm pore size; Corning) at the amount of 1 × 10^5 cells and preincubated with oligomycin (100 ng/ml), rotenone (0.1 μM), 2-DG (2 mM), and FCCP (5 μM) for 20 min. ATP, MeSADP, or adenosine (500 μM each) was added to the lower compartment to initiate migration. After 24 h, nonmigrated cells were scraped off from the top of the filters; migrated cells were fixed in methanol for 15 min at room temperature and stained with 0.2% crystal violet in 2% ethanol for 15 min. Migrated cells were photographed under ×20 magnifications in a phase-contrast microscope (Nikon) at three to five random fields. The number of tubes and average tube length were determined using ImageJ64 analysis.

**Cell culture for bioenergetic analysis.** VVEC were split into two 60-mm petri dishes containing complete growth media (DMEM, 10% FBS) for 1 h to allow for cell attachment and changed to growth medium containing 25 mM glucose or 20 mM galactose + 5 mM glucose thereafter. Cells were maintained for 36 h at 37°C and 5% CO2 until 90–95% confluence was reached. On reaching optimal confluence, cells were trypsinized and seeded in Seahorse XF 24-well cell culture plates (Seahorse Bioscience, Billerica, MA) at 2.0 × 10^4 cells/well in HBSS in the absence of sodium bicarbonate, calcium, and magnesium (Corning, One Riverfront Plaza, NY). Seeding was performed using a two-step process in an Matriplat Matrix (BD Bioscience). Growth-arrested VVEC were seeded on polymerized Matriplat in triplicate at a density of 1.7 × 10^4 cells/well and preincubated with oligomycin (100 ng/ml), rotenone (0.1 μM), 2-DG (2 mM), and FCCP (5 μM) for 20 min before stimulation with ATP, MeSADP, or adenosine (100 μM each). Images were captured after 6–8 h using a digital camera connected to a phase-contrast microscope (Nikon) at three to five random fields. The number of tubes and average tube length were determined using ImageJ64 analysis.

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was eliminated by centrifugation for 10 min at 12,000 g. pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. Cellular debris orthovanadate, 10 mM dithiothreitol, 10 mM glycerophosphate, 10 mM NaF, 1 mM sodium EDTA, 10% glycerol, 1% Nonidet P-40, 1% Triton, 1 mM incubation, cells were washed with cold PBS and lysed on ice for 30 min, 4 h, and 24 h. When PI3K inhibitor (LY294002) or Akt serum, 72 h) were stimulated with ATP, MeSADP, or Ado (100 g/ml leupeptin, 10 g/ml aprotinin, 10 g/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. Cellular debris was eliminated by centrifugation for 10 min at 12,000 g. Equivalent amounts of total cell protein (20 g) were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (GE Health Care, Pittsburgh, PA). Membranes were blocked in PBS containing 0.1% Tween 20 and 1% BSA for 1 h at room temperature, and incubated with primary antibodies overnight at 4°C, and with secondary antibodies for 1 h at room temperature. The following primary antibodies were used: phospho-Akt, Akt, hexokinase (HK) 2 (1:1,000, Cell Signaling Technology, Danvers, MA); phospho-lactate dehydrogenase (LDH) (1:500; Cell Signaling Technology), LDH (1:1,000; Abcam, Cambridge, MA); pyruvate dehydrogenase (PDH), phospho-PDH (1:2,000; Abcam); phospho-PFKB3 (1:000; Aviva Systems Biology, San Diego, CA); PFKB3 (1:2,000; Abcam); succinate dehydrogenase (SDH) (1:1,000; Abcam); cytochrome oxidase subunit IV (COX IV) and F1F0 ATP synthase β-subunit (1:000; Santa Cruz Biotechnology, Santa Cruz, CA), and GLUT-1 (1:1,000; Abcam). All antibiotics were diluted in PBS containing 0.1% Tween 20 and 1% BSA. Immunoactive bands were detected using ECL kit (Renaissance, NEN Life Science Product), followed by exposure to Optimum X-ray Film (Life Science Products, Frederick, CO). In all experiments, equal sample loading and transfer were verified by staining nitrocellulose membranes with Ponceau solution and by probing with anti-GAPDH antibodies (dilution 1:1,000; Cell Signaling Technology). Calculations of the protein band densities were performed using ImageJ software.

GLUT-1 immunoﬂuorescence. VVEC were plated on eight-well chamber slides (Labtek, Grand Rapids, MI), serum starved, and stimulated with ATP (100 µM) for 0.5–24 h. Stimulated cells were washed three times with PBS (5 min each) and blocked in PBS containing 5% normal goat serum and 0.3% Triton-X 1 h at room temperature. Cells were incubated with rabbit polyclonal antibody against GLUT-1 (Abcam no. 115730; 1:400) overnight at 4°C. After the incubation, cells were washed as indicated above and incubated with fluorescein-conjugated Grifonia Simplicifolia lectin (Vector Labs, Burlingame, CA; no. FL-1101) at the concentration of 2 µg/ml for 1 h at room temperature. Following this step, cells were washed and incubated with the secondary, goat anti-rabbit antibody Alexa Fluor-594 (Jackson Labs, Farmington, CT; no. 111-585-003; 1:400), for 1 h at room temperature. Finally, cells were washed, air dry in the dark, and treated with Prolong Gold anti-fade with 4,6-diamidino-2-phenylindole (Cell Signaling Technology, no. 8961-I). Images were captured using EVOS imaging system under ×40 magnification.

Measurement of lactate and ATP production. Cells were grown and stimulated as described above for Western blot analysis. Aliquots of total cell lysates (30 µl) were analyzed with l-Lactate Assay Kit (Cayman Chemical, Ann Arbor, MI). For ATP measurements, 300 µl of conditioned media were collected into chilled polypropylene tubes (Sigma, St. Louis, MO) and centrifuged at 12,000 g for 10 min to remove any cell debris. Media were frozen at −80°C for subsequent measurement. ATP sample concentrations were detected with the luciferase luciferin kit (ENLITEN ATP Assay System, Promega, Madison, WI). Both lactate and ATP measurements were performed using a Polstar Omega microplate reader (BMG Labtech, Cary, NC). The samples were compared with a lactate or ATP standard curve performed in each individual experiment.

Intracellular Ca2+ measurements. VVEC were cultured in glass-bottom dishes suitable for fluorescent imaging (MatTek, Ashland, MA) and growth-arrested in serum-free DMEM for 72 h before the experiments. Immediately before the Ca2+ measurement, cells were incubated in the presence of membrane-permeable Ca2+ indicator rhodamine 2AM (Invitrogen R-1245MP; red color in Fig. 7A) at 1 µM for 30 min at room temperature, washed with media, and left to recover for 10 min to reduce the spontaneous intrinsic Ca2+ activity. In some experiments, cells were pretreated with the inhibitor of Ca2+ transport to mitochondria, ruthenium red (1 µM, 30 min). Time-lapse image acquisition was started, and, after establishing the baseline for 150 s, the acquisition was paused to perfuse the cell culture dish with media containing 100 µM ATP. Image acquisition was resumed immediately after the perfusion. To determine Ca2+ signal localization to mitochondria, Ca2+ influx was assayed in live cells coloaded with MitoTracker Deep Red FM (Invitrogen M22426) at 0.8 µM for 30 min at 37°C and with rhodamine 2AM (red and green, respectively, in Fig. 7B). In both experimental settings, the time-lapse images were captured with 10-s intervals on Nikon TE2000 microscope equipped with Cooke CCD SensiCam. SlideBook software was used for image acquisition and analysis. Averaged single-cell Ca2+ traces were measured by fluorescence intensity levels within hand-drawn regions of interest in individual cells (n = 15–20).

Statistical analysis. For the analysis of variances between groups of data, two-way ANOVA with a Bonferroni posttest was performed using GraphPad Prism 4.0. Data are expressed as means ± SE. A P value of <0.05 was considered statistically significant. Three to five individual experiments were carried out for each assay. VVEC populations were isolated from at least three different animals.

RESULTS

VVEC growth and angiogenesis requires both glycolysis and OXPHOS. To determine the role of glycolysis and OXPHOS in VVEC proliferative responses, we used pharmacological inhibitors that target different steps of cellular energy production. Our data show that treatment of VVEC with glycolytic inhibitor 2-DG (glucose analog that inhibits HK), and the inhibitors of mitochondrial complex I (rotenone) and complex V (oligo-
mycin), significantly decreased cell proliferation rate (Fig. 1A). Following this observation, the effects of metabolic inhibitors were evaluated on purinergic receptor-mediated angiogenic responses. Extracellular ATP and the stable ADP analog MeSADP (both P2R agonists) were used in this study based on our laboratory’s previously published data showing pro-angiogenic effect of these agonists in VVEC (30, 46, 80), whereas the effects of extracellular adenosine (P1R agonist) on angiogenic and metabolic responses have not been previously investigated. Treatment with ATP, MeSADP, and adenosine robustly increased VVEC DNA synthesis (Fig. 1B). 2-DG, rotenone, and oligomycin, as well as mitochondrial uncoupler, FCCP, significantly decreased the rate of DNA synthesis in cells exposed to all three purinergic receptor agonists. The more dramatic inhibitory effect was observed in response to 2-DG and FCCP compared with

Fig. 1. Glycolysis and mitochondrial oxidative phosphorylation are necessary for VVEC proliferative and migratory responses. A: proliferation rates were determined in VVEC grown in DMEM/1% FBS in the presence of oligomycin (Oligo; 10 ng/ml), rotenone (Rot; 0.1 μM), 2-deoxyglucose (2-DG; 0.2 mM), and FCCP (0.5 μM) The media with indicated inhibitors were refreshed each second day. Cell proliferation rate was assessed using a fluorescent CyQuant proliferation kit. Values are means ± SE from 3 independent experiments. B: [3H]thymidine incorporation was determined in growth-arrested VVEC (DMEM without serum, 72 h) in response to stimulation with extracellular ATP (100 μM). Cells remained untreated or were treated with 2-DG (2 mM), Rot (0.1 μM), Oligo (100 ng/ml), or FCCP (2 μM) 20 min before stimulation. Values are means ± SE from 3 independent experiments. ###P < 0.001, control vs. ATP-stimulated cells. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with ATP-treated cells. C: for migration assay, growth-arrested cells were seeded in Boyden chamber-permeable inserts and pretreated with the indicated for 20 min. ATP, MeSADP, or adenosine (Ado; 500 μM) were added to the lower compartment to initiate migration. Number of migrated cells was evaluated as described in METHODS. Values are means ± SE from 4 independent experiments. ###P < 0.001, control vs. ATP-stimulated cells. *P < 0.05, **P < 0.01, and ***P < 0.001, untreated vs. treated with inhibitors.
rotenone and oligomycin, under both basal and nucleotide-stimulated conditions.

Furthermore, a role of cellular energy pathways in VVEC angiogenesis was determined in cell migration and tube formation assays. As shown in Fig. 1C, ATP and MeSADP were almost equipotent in stimulating VVEC migration. In contrast, adenosine was not effective, indicating that P1Rs do not mediate the migration response. The responses to ATP and MeSADP were significantly reduced by 2-DG (by 30–50%) and rotenone (by 50–60%) and even more dramatically reduced by oligomycin (by 70–75%) and FCCP (66–72%).

Tube formation assay on growth factor reduced Matrigel demonstrated VVEC rearrangement into tubular-like network in response to ATP and MeSADP, but not to adenosine (Fig. 2). The observed tube formation response was evaluated as increase in tube length and decrease in number of tubes in nucleotide-stimulated cell compared with control, either treated or untreated with inhibitors. Rotenone, oligomycin, and FCCP prevented tube formation; however, 2-DG did not show this effect. Similar inhibitory effects on tube formation were observed when cells were stimulated with MeSADP. Together, these studies demonstrated that both glycolysis and OXPHOS play a critical role in angiogenic responses in VVEC, and purinergic receptor agonists show diverse potency in mediating proliferation, migration, and tube formation.

P2Rs stimulate glycolysis in VVEC. To examine if purinergic receptor stimulation activates VVEC glycolysis, we used the Seahorse Bioscience XF24 Extracellular Flux Analyzer, which measures glycolytic response as ECAR in intact cells and enables dynamic treatment of cells through injector ports. For the ECAR experiments, VVEC were cultured in a standard growth medium with 25 mM glucose and incubated with unbuffered Seahorse XF Base medium without glucose 1 h before metabolic flux measurements. Stimulation with extracellular ATP and MeSADP resulted in a dramatic increase in ECAR by 3.3- and 1.9-fold, respectively, in VVEC (Fig. 3, A)

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**Fig. 2. Effects of metabolic inhibitors on purinergic receptor-mediated VVEC tube formation.** Growth-arrested VVEC were seeded in angiogenesis slides (ibidi) covered with polymerized Growth Factor Reduced Matrigel, as described in METHODS. Cells (1.7 × 10⁴ cells/well) were pretreated with 2-deoxyglucose (2-DG; 2 mM), rotenone (Rot; 0.1 μM), oligomycin (Oligo; 100 ng/ml), or FCCP (2 μM) for 20 min, followed by stimulation with ATP, MeSADP, or adenosine (Ado; 100 μM) for 6 h. Images were captured using a digital camera connected to a phase-contrast microscope (Nikon). Top: shown are representative images from one of five experiments performed on three VVEC populations. Bottom: tube formation responses were characterized by evaluation of number of tubes and average tube length, using ImageJ64 program. Values are means ± SE.
In contrast, stimulation with extracellular adenosine did not have any effect on ECAR. Injection of oligomycin resulted in a biphasic ECAR response (a decrease followed by an increase), but no additional increases above the nucleotide- and glucose-stimulated level were observed. The ECAR response was muted by the addition of glycolytic inhibitor 2-DG, and cells tended to maintain ECAR above baseline. To complement these data, we evaluated the effects of purinergic receptor agonists on lactate production. As shown in Fig. 3C, ATP and MeSADP stimulated lactate production by 2.4- and 1.45-fold, respectively. In contrast, adenosine was ineffective, indicating that P2R, but not P1R, are positive regulators of VVEC glycolysis.

The upregulation of glycolysis prompted us to examine the effects of purinergic receptor activation on the expression level and/or activation of key glycolytic enzymes. Stimulation with extracellular ATP, MeSADP, and, to a lesser degree, adenosine increased HK2 expression at 30 min of incubation. The effects of ATP and MeSADP, but not adenosine, were observed on HK2 expression level at 24 h of incubation (Fig. 4A). Under the same conditions, ATP increased the phospho-PFKB3 level at 4 h, and MeSADP exerted this effect at 4 and 24 h. Stimulation with adenosine did not increase phospho-PFKB3 level (Fig. 4B). We also found that ATP slightly increased the expression of LDH that was observed after 24 h of stimulation; however, MeSADP did not show this effect (Fig. 4C). Moreover, stimulation adenosine decreased the LDH level by about twofold compared with the basal level.

Fig. 3. Extracellular nucleotides (nucl) ATP and MeSADP, but not adenosine (Ado), stimulate aerobic glycolysis in VVEC. A: glycolytic rate was measured as extracellular acidification rate (ECAR) using XF24 Extracellular Flux Analyzer (Seahorse Bioscience), as described in METHODS. ATP, MeSADP, or Ado (100 μM each), glucose (Gluc; 10 mM), oligomycin (Oligo; 0.8 μM), or 2-DG (10 mM) were injected at the indicated times. B: data show ECAR rates measured between 15 and 35 min of each agonist treatment. Values are means ± SE from 4 independent experiments. ***P < 0.01, nonstimulated vs. agonist-stimulated cells. C: lactate was determined in a conditioned media of VVEC stimulated with ATP, MeSADP, or Ado for indicated times, as described in METHODS. The average basal lactate production level was 23.9 ± 3.3 μM. Values are means ± SE from 3–5 independent experiments. **P < 0.01 and *P < 0.05, nonstimulated vs. agonist-stimulated cells. Cont, control.
Considering that glucose is a primary energy source for cellular energy pathways, we also examined the effects of purinergic receptor agonists on GLUT-1 expression, a facilitative glucose transporter known to play a critical role in glucose uptake in EC (66). Using Western blot analysis (Fig. 5A), we found that stimulation with extracellular ATP (100 μM) significantly increased GLUT-1 expression, which was observed between 30 min and 4 h, with a maximal response at 1.5 h. MeSADP was as potent as ATP, but the effect of adenosine was less significant (data not shown). We also found that stimulation with extracellular ATP results in a GLUT-1 translocation to the plasma membrane. In these experiments, VVEC were simultaneously labeled with fluorescein-conjugated *Grifonia Simplicifolia* lectin and immunoprobod with anti-GLUT-1 antibodies (Fig. 5B).

**OXPHOS in VVEC is regulated by purinergic receptors and galactose.** Our data presented in Figs. 1 and 2 strongly indicate that OXPHOS is necessary for VVEC angiogenic responses. Although regulation of mitochondrial OXPHOS via signaling mechanisms and various metabolic substrates has been demonstrated in several cell types, the data on EC are limited. Using Seahorse analyzer, we examined if OXPHOS (measured as OCR) can be regulated by extracellular nucleotides and adenosine. Since galactose was reported to increase oxidative capacity in different cell types (3, 41), we also investigated the possible modulation OXPHOS in VVEC by glucose and galactose. Culturing VVEC in the presence of 20 mM galactose and 5 mM glucose for 7 days increased basal OCR, compared with 25 mM glucose (Fig. 6). Galactose culture condition also resulted in a significant increase in maximal OCR rate (FCCP response, or State 3 uncoupled). Treatment with oligomycin resulted in near equivalent decreases in OCR in cells cultured in both high-galactose and high-glucose medium, indicating near equivalent ATP production via OXPHOS in mitochondria under conditions tested. Treatment with rotenone and antimycin A (complex I and III inhibitors, respectively) resulted in OCR decrease below the oligomycin-treated level, suggesting a negligible oligomycin-insensitive respiration in VVEC under both high-glucose and high-

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![Fig. 4. Extracellular ATP, MeSADP, and adenosine (Ado) upregulate key glycolytic enzymes in VVEC. Growth-arrested cells were stimulated with ATP, MeSADP, or Ado (100 μM each). HK, phospho-PFKB3, and lactate dehydrogenase (LDH) were determined in total cell lysates by Western blot analysis. Values are means ± SE from 3–5 independent experiments performed on 3 cell populations. **P < 0.01 and *P < 0.05, nonstimulated vs. agonist-stimulated cells.](http://ajpcell.physiology.org/)

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![Fig. 5A](http://ajpcell.physiology.org/)

![Fig. 5B](http://ajpcell.physiology.org/)
Extracellular ATP elevates mitochondrial Ca\(^{2+}\) and upregulates mitochondrial metabolic enzymes. Elevation of intracellular Ca\(^{2+}\) is physiologically important for both the regulation of OXPHOS and cell proliferation (33, 46, 57). Moreover, our laboratory’s previous study demonstrated that P2Y\(_1\) and P2Y\(_{13}\)-mediated mitogenic signaling in VVEC are Ca\(^{2+}\) dependent, but purinergic regulation of mitochondrial Ca\(^{2+}\) in VVEC remain unexplored. To assess this possibility, mitochondrial Ca\(^{2+}\) responses were determined in VVEC loaded with membrane-permeable Ca\(^{2+}\) indicator rhodamine 2AM (Fig. 7). The maximal response was observed at 150 s after stimulation and declined by 420 s (Fig. 7, A and C). Loading cells with rhodamine 2AM and Mitotracker DeepRed allowed simultaneous visualization of Ca\(^{2+}\) signal and mitochondria (Fig. 7B). The observed ATP-induced Ca\(^{2+}\) increase was attenuated by preincubation (1 \(\mu M\), 30 min), with a specific inhibitor of Ca\(^{2+}\) transport to mitochondria, ruthenium red (Fig. 7D), demonstrating mitochondrial localization of the signal.

As OXPHOS is important for VVEC angiogenesis and is regulated via purinergic receptors, we next examined whether purinergic receptor agonists modulate the expression of mitochondrial enzymes. As shown in Fig. 8A, stimulation with extracellular ATP, MeSADP, and adenosine for 24 h decreased phosphorylation of the PDH-E1\(\alpha\), indicating its activation. We also observed significant increase in the expression level of COX IV, after 4 h exposure, to indicated purinergic receptor agonists (Fig. 8B). In addition, MeSADP and adenosine, but not ATP, slightly increased the expression of SDH (Fig. 8C) and F\(_{1}\)F\(_{0}\) ATP synthase \(\beta\)-subunit (Fig. 8D) observed at 24 and 4 h, respectively. In contrast, adenosine downregulated F\(_{1}\)F\(_{0}\) ATP synthase \(\beta\)-subunit expression at 24 h.

**PI3K and Akt are involved in the regulation of VVEC metabolic pathways.** PI3K/Akt pathway plays an important role in cell proliferation, growth factor-induced glycolysis, and mitochondrial biogenesis (16, 78, 81). Therefore, we examined the effects of PI3K and Akt inhibitors on lactate production and the expression of metabolic enzymes. Treatment with extracellular ATP, MeSADP, and adenosine induced a biphasic Akt phosphorylation with an early response observed at 30 min, and a later increase at 4 h (Fig. 9A). Pretreatment with PI3K inhibitor, LY294002 (10 \(\mu M\)), significantly decreased the basal and ATP-stimulated lactate levels by 44% and 67%, respectively. Pretreatment with an Akt inhibitor, GSK690693 (10 nM), attenuated ATP-stimulated lactate production by \(\sim 17\%\) (Fig. 9B), indicating the involvement of Akt and possibly another intracellular kinase pathways, in the regulation of glycolysis in VVEC. We also demonstrated that pretreatment with LY294002 decreased phospho-Akt (Fig. 9C), and both LY294002 and GSK690693, although to a different extent, decreased the levels of HK, phospho-PFK3, LDH, SDH, and GLUT-1 in ATP-stimulated cells (Table 1).

**DISCUSSION**

Angiogenesis of the VV plays a pathological role in the development of hypoxia-induced PH. In the present study, using VVEC isolated from PA VV, we extended our previous work to evaluate a possible role of cellular energy pathways in VVEC angiogenic responses and regulation of these pathways
Fig. 6. Extracellular nucleotides, adenosine (Ado), and galactose (Gal) increase respiratory capacity of VVEC. Mitochondrial respiration (oxygen consumption rate [OCR]) analysis was performed using XF24 Extracellular Flux Analyzer in VVEC cultured in media containing either 25 mM glucose (Glu) or 5 mM Glu and 20 mM Gal, as described in METHODS. A–D: control (A), ATP (B), MeSADP (C), Ado (D), oligomycin (Oligo; 0.8 μM), FCCP (2 μM), rotenone (Rot; 0.1 μM), and antimycin A (AA; 2 μM) were injected at the indicated times. E and F: data on basal and maximum respiratory rates represent means ± SE from 6–8 independent experiments performed on 4 cell populations. ***P < 0.001, Gal- vs. Glu-containing medium. a|P < 0.05, a|P < 0.01, and a|P < 0.001, nonstimulated vs. nucleotide- or Ado-stimulated cells.
Our study revealed distinct roles of P2R (ATP and MeSADP) and P1R (adenosine) in VVEC angiogenic and metabolic responses. We found that glycolysis and OXPHOS are both necessary for VVEC angiogenesis, suggesting that VVEC may represent a “bivalent” metabolic phenotype with a greater reliance on glycolysis in response to P2R stimulation. In addition, consistent with a previously reported role of PI3K and Akt pathways in VVEC proliferative responses (30, 80), our study demonstrated a role of the PI3K/Akt signaling pathway in the induction of several key glycolytic and OXPHOS enzymes, as well as the GLUT-1 transporter.

The relationship between cellular metabolism and proliferative responses has been investigated in various cell types. Preference for aerobic glycolysis is considered to be a characteristic feature of tumor cells and other cell types with a highly proliferative potential (44, 72). Increase in proliferative and angiogenic activity is accompanied by utilization of glucose (32), increase of LDH activity (51, 52), and activation of glycolysis (2, 22, 23). In the present study using 2-DG, rotenone, oligomycin, and FCCP, we evaluated a relative contribution of OXPHOS and glycolysis on purinergic receptor-mediated VVEC angiogenesis. Consistent with previous reports showing involvement of the glycolytic pathway to angiogenic potential, we found that treatment with 2-DG attenuates both P1R and P2R agonists-stimulated VVEC DNA synthesis and P2R-stimulated migration. Contrary to our expectations, treatment with 2-DG did not affect nucleotide-mediated tube formation on Matrigel, suggesting that OXPHOS may be relatively more important for tube formation response. Our observations on the role of glycolysis in VVEC angiogenesis are in agreement with recent findings for a critical role of glycolysis in vessel sprouting during retinal angiogenesis (22, 83). Noteworthy, in this model, VEGFR2-increased expression of PFKFB3 leads to localized glycolytic ATP production to lamelopodia and filopodia (23). Our data are also in agreement with the previous studies that demonstrated that PA EC from patients with idiopathic pulmonary arterial hypertension exhibit abnormal metabolic phenotype characterized by increased proliferative responses, which is accompanied by resistance to apoptosis and metabolic switch to glycolysis (82).

Regulation of glycolysis through the receptor-dependent mechanisms has been reported for several pro-angiogenic factors, including VEGF, endothelin-1, IL-1β, TNF-α, and ATP (6, 9, 71, 73, 77). Although some studies postulated coordinated regulation of metabolic and signaling activities, regula-
tion on cellular energy pathways by extracellular nucleotides in the context of angiogenesis has not been previously investigated. Here, we present evidence that P2R agonists, ATP and MeSADP, but not P1R agonist, adenosine, upregulate VVEC glycolytic activity. This response was evaluated as increased lactate production, increase in ECAR, and stimulation of PFKB3 and HK2, enzymes that represent the essential control points in glycolysis (Fig. 10). Despite increased lactate production, we did not observe any significant effects of purinergic receptor agonists on LDH expression, suggesting that enzymatic activity, but not the enzyme level, is induced by P2R agonists. In addition, our study demonstrates P2R-mediated upregulation and plasma membrane translocation of the facilitated glucose transporter GLUT-1. In agreement with our findings, others have shown P2R-mediated GLUT-1 and GLUT-4 translocation in C2C12 skeletal muscle cells and 3T3-L1 adipocytes, as well as VEGF-mediated GLUT-1 translocation to the plasma membrane in retinal EC (8, 42, 66). Moreover, transfection of P2X7 in human embryonic kidney-293 cells resulted in serum-independent growth, upregulation of glycolytic enzymes, GLUT-1, and increase of mitochondrial Ca$^{2+}$ (1, 6). Therefore, together with previous findings, our study presents evidence for the role of purinergic control of glycolysis coupled to angiogenic activation of VVEC.

The importance of mitochondria and oxidative metabolism has been demonstrated in oncogenic transformation (31, 67), cell differentiation (54), neo-angiogenesis (18), and metabolic adaptation (29, 34, 38, 79), indicating that glycolysis is not exclusive to energy production, especially in slow-growing tumors (reviewed in Ref. 40). Some cancer cells maintain mitochondrial function to rapidly switch from glycolysis to OXPHOS during carcinogenesis (65). Studies on skeletal muscle and retina demonstrate the importance of mitochondrial biogenesis for the angiogenic response mediated by VEGF-1/ peroxisome proliferator-activated receptor-α coactivator-1 (PGC-1α) pathway (60, 61). Using rotenone (complex I inhibitor) and oligomycin (an inhibitor of F$_{1}$F$_{0}$ ATP synthase), we demonstrated a critical role of OXPHOS in VVEC angiogenesis. Rotenone and oligomycin were almost equipotent in downregulating DNA synthesis, migration, and tube formation, while rotenone had more dramatic inhibitory effect on DNA synthesis compared with migration. Notably, the inhibitory effects of rotenone and oligomycin indicate that the glycolytic pathway cannot fully maintain VVEC angiogenic capabilities,
suggesting mitochondrial activity is necessary to maintain VVEC responses. In all studies, mitochondrial membrane potential (ΔΨm) uncoupler FCCP had a potent inhibitory effect on VVEC angiogenesis, including tube formation, which is considered a later, differentiation-associated angiogenic response.

Table 1. Effect of PI3K and Akt inhibitors on ATP-stimulated metabolic enzyme expression in VVEC

<table>
<thead>
<tr>
<th>Metabolic Proteins</th>
<th>LY294002 (10 μM)</th>
<th>GSK690693 (10 nM)</th>
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<tr>
<td>HK</td>
<td>62.5 ± 0.9a</td>
<td>40.7 ± 9.3a</td>
</tr>
<tr>
<td>p-PFKB3</td>
<td>53.0 ± 10.4b</td>
<td>76.1 ± 9.9b</td>
</tr>
<tr>
<td>LDH</td>
<td>108 ± 1.0</td>
<td>133.2 ± 21.5c</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>30.1 ± 14.6b</td>
<td>29.3 ± 12.6b</td>
</tr>
<tr>
<td>SDH</td>
<td>72.5 ± 4.9b</td>
<td>55.2 ± 2.1b</td>
</tr>
<tr>
<td>p-Akt</td>
<td>48.0 ± 14.1c</td>
<td>168.2 ± 62.1</td>
</tr>
</tbody>
</table>

Values are means ± SE in % of ATP-stimulated level. Growth-arrested cells (72 h, without serum) were preincubated with LY294002 (10 μM) or GSK690693 (10 nM) for 30 min, or remain untreated and stimulated with 100 μM ATP for 24 h. The expression of indicated metabolic proteins was determined by Western blot analysis, as described in METHODS. The calculated values from 3–4 independent experiments represent the percentage of indicated protein expression in LY294002- and GSK690693-treated cells compared with the expression in ATP-stimulated cells (taken as 100%). *P < 0.001, **P < 0.01, and ***P < 0.05.

Consistent with our observation, data from tumor angiogenesis and wound-healing models demonstrate that the mitochondrial uncoupler embelin impairs neo-angiogenesis by selectively targeting proliferation of EC (18). It was also demonstrated that ΔΨm, the predominant component of an electrochemical gradient, plays an important role in differentiation of neuroblastoma and embryonic stem cells (47, 74), indicating that ΔΨm may have a role in regulation of cellular responses. Therefore, despite the common acceptance of EC as a “glycolytic” cell type, our study and those of others (20, 25, 64) prove that mitochondria function is required for angiogenesis and serve to further characterize EC phenotypes from both quiescent and angiogenic vessels.

The data on the role of OXPHOS and its regulation by extracellular signals are in agreement with several previous studies that link proliferative responses and mitochondrial function. For example, PGC-1α-mediated mitochondrial biogenesis is important for VEGF- and exercise-stimulated angiogenic responses in skeletal muscle and retina (7, 17, 61, 81). Fibroblast growth factor 21 was shown to regulate energy metabolism by activating AMP-activated protein kinase-sirtuin 1-PGC-1α in the adipocyte 3T3-L1 line (15). In addition,
recent studies demonstrated insulin-dependent regulation of OXPHOS, mitochondrial protein expression, and ATP production in skeletal muscle (10, 11, 70). In line with these observations, our study demonstrates that P2R agonists, ATP and MeSADP (and to a lesser extent, P1R agonist adenosine), induce both basal and maximal OCR and upregulate proteins critically involved in oxidative metabolism, i.e., PDH, SDH, COX IV, and \( \beta \)-subunit of mitochondrial \( \mathrm{F}_1\mathrm{F}_0 \) ATP synthase. Furthermore, it was previously shown that OXPHOS can be regulated by substrate availability (37), and that skeletal muscle response to galactose is associated with increased mitochondrial biogenesis (3). We found that culturing VVEC in the presence of 20 mM galactose and low glucose increased basal and purinergic agonist-induced basal and maximal OCR, indicating a shift of cellular metabolism toward a more oxidative one. This effect of extracellular nucleotides was potentiated in cells grown in the presence of galactose, suggesting that mitochondrial function can be regulated metabolically and via receptor-mediated signaling pathways.

Intracellular and mitochondrial Ca\( ^{2+} \) homeostasis plays an important role in metabolic and proliferative signaling (33, 57). Although mitochondria have emerged as important targets of agonist-dependent Ca\( ^{2+} \) elevation (33, 35, 39), data on purinergic regulation of mitochondrial Ca\( ^{2+} \) responses are very limited (85, 86). It was established that increased mitochondrial Ca\( ^{2+} \) results in increased OXPHOS via the activation of Ca\( ^{2+} \)-sensitive dehydrogenases, leading to accumulation of reduced cofactors (NADH, FADH\(_2\)) necessary for \( \Delta \nu_{\text{m}} \) and ATP synthesis (33). Our laboratory’s previous studies demonstrated a role of P2Y\(_i\)R and P2Y\(_i\)R in the elevation of intracellular Ca\( ^{2+} \) coupled to the activation of mitogenic signaling pathways in VVEC (46). In the present study, we demonstrated that extracellular ATP (P2YR agonist) elevates mitochondrial Ca\( ^{2+} \) in VVEC. This observation is consistent with other reports demonstrating an increase in mitochondrial Ca\( ^{2+} \) in response to purinergic stimulation of astrocytes and Sertoli cells (75, 86). Increase of OCR rate and glucose uptake in response to Ca\( ^{2+} \) mobilizing hormones has also been demonstrated in hepatocytes and mononuclear cells (43, 45). Taken together, our data support the idea of receptor-dependent regulation of mitochondria and a role of mitochondria in VVEC angiogenic response (Fig. 10).

We previously reported that the PI3K/Akt pathway is critical for ATP-induced angiogenic responses in VVEC (30, 80). Our present study implicates PI3K/Akt pathway in the angiogenic regulation of glucose uptake, and cellular energy metabolism in VVEC. Notably, we found biphasic (around 30 min and 24 h) response of Akt phosphorylation in ATP, MeSADP, and adenosine-treated cells. This response can be explained by the involvement of intracellular network of PI3K, PTEN (phosphatase and tensin homolog), PPI, and mammalian target of rapamycin (mTOR) C2 pathways that regulate time-dependent phosphorylation and dephosphorylation signals (12, 13, 48, 59). Activation of these pathways, as well as an autocrine positive feedback signaling via ATP and cytokine release from VVEC, cannot be excluded and may explain a secondary (24 h) response of Akt phosphorylation in VVEC.

We also demonstrate that P2R-induced lactate production, phosphorylation of PFK3, and upregulation of HK and SDH are all sensitive to PI3K inhibitor LY294002 and, to a lesser extent, to Akt inhibitor, GSK690693, suggesting that PI3K may play a central role in the glycolysis regulation, whereas there might be additional kinase pathways, in addition to Akt, responsible for the regulation of GLUT-1 and/or glycolytic enzymes (Fig. 10). In line with our observations on metabolic involvement of PI3K/Akt pathway, insulin-induced Akt translocation to mitochondria has been shown to stimulate PDH activity and mitochondrial ATP production in cardiomyocytes (24, 84). Akt and mTOR have been demonstrated to regulate mitochondrial activity via the activation Yin-Yang 1 and PGC-1\( \alpha \) transcription factors (19, 63), pointing out the importance of the Akt/mTOR pathway for cellular energy pathways.

Collectively, the results from our study present new evidence that VVEC angiogenesis requires functional glycolysis and OXPHOS cellular energy pathways that can be modulated by purinergic receptor activation and metabolic substrates. Given the autocrine/paracrine role of extracellular ATP in hypoxia-induced VV angiogenesis, the results from our study may suggest development of new therapeutic strategies to limit pathological angiogenesis in cardiovascular diseases via simultaneous targeting of purinergic receptors and cellular metabolic pathways.

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Present address of T. Lyubchenko: College of Health Sciences, University of Massachusetts Lowell, Weed Hall 218, 3 Solomont Way, Lowell, MA 01854.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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