Critical role for lactate dehydrogenase A in aerobic glycolysis that sustains pulmonary microvascular endothelial cell proliferation

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Submitted 13 August 2009; accepted in final form 27 July 2010

Parra-Bonilla G, Alvarez DF, Al-Mehdi AB, Alexeyev M, Stevens T. Critical role for lactate dehydrogenase A in aerobic glycolysis that sustains pulmonary microvascular endothelial cell proliferation. Am J Physiol Lung Cell Mol Physiol 299:L513–L522, 2010. First published July 30, 2010; doi:10.1152/ajplung.00274.2009.—Pulmonary microvascular endothelial cells possess both highly proliferative and angiogenic capacities, yet it is unclear how these cells sustain the metabolic requirements essential for such growth. Rapidly proliferating cells rely on aerobic glycolysis to sustain growth, which is characterized by glucose consumption, glucose fermentation to lactate, and lactic acidosis, all in the presence of sufficient oxygen concentrations. Lactate dehydrogenase A converts pyruvate to lactate necessary to sustain rapid flux through glycolysis. We therefore tested the hypothesis that pulmonary microvascular endothelial cells express lactate dehydrogenase A necessary to utilize aerobic glycolysis and support their growth. Pulmonary microvascular endothelial cell (PMVEC) growth curves were conducted over a 7-day period. PMVECs consumed glucose, converted glucose into lactate, and acidified the media. Restricting extracellular glucose abolished the lactic acidosis and reduced PMVEC growth, as did replacing glucose with galactose. In contrast, slow-growing pulmonary artery endothelial cells (PAECs) minimally consumed glucose and did not develop a lactic acidosis throughout the growth curve. Oxygen consumption was twofold higher in PAECs than in PMVECs, yet total cellular ATP concentrations were twofold higher in PMVECs. Glucose transporter 1, hexokinase-2, and lactate dehydrogenase A were all upregulated in PMVECs compared with their macrovascular counterparts. Inhibiting lactate dehydrogenase A activity and expression prevented lactic acidosis and reduced PMVEC growth. Thus PMVECs utilize aerobic glycolysis to sustain their rapid growth rates, which is dependent on lactate dehydrogenase A.

angiogenesis; progenitor cells; repair

CELLS REQUIRE ATP to sustain their bioenergetic demands. Glucose is a principal carbon substrate needed to generate cellular ATP. Glycolysis in the cytosol of the cell converts glucose into 2 pyruvate molecules and in so doing generates 2 ATP molecules. Pyruvate uptake into mitochondria results in its complete oxidation and enables conversion of oxidation-derived energy into 36 ATP. Oxidation-derived energy requires oxygen delivery to the mitochondria, and since this mode of ATP synthesis is highly efficient, mammalian cells principally use mitochondria to sustain their bioenergetic demands (37). However, in some instances, even in the presence of sufficient oxygen, cells utilize glycolysis to meet their ATP requirements through a process referred to as aerobic glycolysis (36). In this case, despite the presence of adequate oxygen to support mitochondrial respiration, glycolytic flux is rapid, and pyruvate is converted to lactate, resulting in pronounced glucose consumption. Many rapidly growing cells, such as cancer cells, switch from traditional mitochondria-dependent metabolic pathways to utilize aerobic glycolysis to sustain their rapid growth (16, 36). Rapidly growing nontransformed cells display a similar metabolic profile, as proliferating lymphocytes (4, 18, 38), thymocytes (5, 13, 14), and fibroblasts (22) utilize aerobic glycolysis. These findings support the general idea that cells undergoing rapid growth utilize aerobic glycolysis as a mechanism to support their bioenergetic demands, yet at present, the growth advantage afforded by use of aerobic glycolysis is poorly understood.

Endothelium is typically quiescent in the healthy postnatal vasculature. Disruption of cell-cell junctions, as occurs during postnatal angiogenesis and in response to vascular injury, triggers a proliferative response that is necessary to support blood vessel development and repair. Capillary endothelium undergoes especially robust angioproliferative responses, both in vivo and in vitro (11, 17, 28, 34). Pulmonary microvascular endothelial cells (PMVECs), for example, are rapidly angiogenic (3, 6). These cells proliferate faster than do pulmonary artery endothelial cells (PAECs), and they are enriched with an extraordinarily high number of replication-competent cells. Based on these criteria, PMVECs represent a population of highly proliferative cells.

It is not presently clear whether PMVECs utilize aerobic glycolysis to support their rapid growth. Endothelial cells, in general, are not thought to rely heavily on mitochondrial metabolism to generate ATP (7, 26). This idea is based in large part on the relatively low number of mitochondria that are found in endothelial cells compared with other metabolically active cell types (1). In addition, prolonged exposure to low oxygen environments does not result in decreased ATP concentrations in endothelium (33, 43). Considering the increased number of highly replication-competent cells, our present studies were undertaken to determine whether PMVECs consume glucose during growth and convert glucose into lactate, indicative of the aerobic glycolysis described in rapidly growing cells. Moreover, since lactate dehydrogenase (LDH) converts pyruvate to lactate, we sought to determine the importance of this enzyme in sustaining aerobic glycolysis and proliferation.

MATERIALS AND METHODS

Isolation and culture of rat lung endothelial cells. Procedures for isolation of rat endothelial cells were approved by the University of South Alabama Institutional Animal Care and Use Committee. Pulmonary microvascular and artery endothelial cells were isolated from male CD40 rats as previously described (8, 32). Whereas PMVECs...
are isolated from vessels ≈25 µm in diameter, mostly reflective of capillaries. PAECs are isolated from the main pulmonary artery and two to three additional vessel branches (12, 19, 23, 31). Cells were seeded at a density of 10^5 cells per well in six-well plates in DMEM (22 mM glucose), 10% FBS, and 1% penicillin-streptomycin. Twenty-four hours after seeding, media was replaced with 22 mM (concentration standard used to culture rat endothelial cells), 11 mM (physiological concentration for rats), or 5 mM glucose (hypoglycemic concentration for rats) or 22 mM galactose-containing media as indicated in RESULTS. In CD40 rats, blood glucose is ~200 mg/dl [Clinical Laboratory Parameters for Crl:CD(SD); Charles River Laboratories, which corresponds to 11 mM. In studies completed monitoring rat blood glucose concentrations, actual glucose measurements range from 10 to 11 mM (T. Stevens, unpublished observations). Solution osmolarity was adjusted to physiological levels.

**Growth curves.** Cells were grown for 7 days at 37°C in 21% oxygen, 5% CO₂ samples were collected from the media every 24 h to make the required measurements (pH, glucose, lactate, and gases oxygen, 5% CO₂) and lactate (millimoles per liter) levels were measured in the medium of all treatments (kept at the same environmental conditions) throughout the growth curves using the YSI 2300 STAT Plus Glucose & Lactate Analyzer (GMI, Yellow Springs, OH).

**pH measurements.** The pH was measured in the medium of all treatments (kept at the same environmental conditions) throughout the growth curves using the ABL 5 blood gas analyzer (Radiometer, Copenhagen, Denmark).

**mRNA expression.** Samples from PMVECs and PAECs grown in either 5 or 22 mM glucose were collected during log-phase growth and at confluence and used to extract RNA. RNA extraction was performed using TRIzol reagent from Invitrogen (Carlsbad, CA) and then purified with an RNAeasy Mini Kit from Qiagen (Austin, TX). RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water, and 100 ng of sample was used for the reaction. iScript One-Step RT-PCR Kit from Bio-Rad was used for the reaction mix, and the primers were as follows: glucose transporter 1 (GLUT-1), 5′-gctctagaaccagttgaagc-3′ and 5′-gagtgtcgttgcttcgca-3′; hexokinase-2 (HK-2), 5′-ctctctccacaggaagtgtaa-3′ and 5′-gctcttcagaeaggtgac-3′; LDH-A, 5′-gaagggcgccagcataa-3′ and 5′-gctcagcaetettccggttc-3′; β-actin, 5′-gtcgcaacctggaggtcata-3′ and 5′-ggtgggttggagtgctcaaa-3′. Quantitative real-time PCR (qRT-PCR) was performed using the iCycler from Bio-Rad. qRT-PCR cycling conditions were as follows: cDNA synthesis at 50°C for 10 min, RT inactivation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 10 s, and primer annealing and extension at 60°C for 30 s. A dissociation curve was generated at 95°C for 1 min and 55°C for 1 min, and the melting curve was generated using 80 cycles at 55°C for 10 s. GLUT-1, HK-2, and LDH-A expression was standardized to that of β-actin.

**Protein levels.** Samples from PMVECs and PAECs grown in either 5 or 22 mM glucose were collected during log-phase growth and at confluence and used to extract total protein. Cells were lysed using a lysis buffer containing 1% SDS, 10% DMSO, and 200 mM sodium acetate. Protein concentration was quantified using a modified Lowry assay from Sigma, and albumin was used to make a standard curve for the assay. Samples were boiled for 5 min, and equal amounts of total protein were loaded into precast SDS-PAGE 4–12% gradient gels from Bio-Rad. The gels were run at 200 V for 1.5 h. Proteins were transferred overnight at 4°C using nitrocellulose membranes from Bio-Rad. Primary antibodies from Santa Cruz Biotechnology.
were used for the immunoblotting of HK-2 (sc-6521) and LDH-A (sc-27230) (1:200 dilution). A primary antibody from Millipore (07-1401) was used for GLUT-1 (1:2,000 dilution). After 2 h of incubation with the primary antibodies, the membrane was incubated with a secondary antibody conjugated with horseradish peroxidase (1:5,000 and 1:10,000 for HK-2 and LDH-A, respectively). Finally, a Pico-Western detection kit from Pierce (SuperSignal West Pico) was used to visualize the bands.

**Oxygen consumption.** Samples from PMVECs and PAECs grown in 22 mM glucose were trypsinized in log-phase, and 10⁶ cells were resuspended in 1.5 ml of fresh medium, placed into a cuvette, and taken to the oxygen meter (Innovative Instruments, Tampa, FL). The 0 and 21% oxygen calibrations were established before sample measurements were made. The electrode was inserted into a sealed cuvette. The reading from the electrode was recorded for 30 min using DASYLab software. The voltage reading was translated into nanomoles of dissolved oxygen at 25°C. Results are reported as nanomoles per minute per 10⁶ cells.

**ATP measurements.** Samples from PMVECs and PAECs were grown in either 5 or 22 mM glucose, collected during log-phase growth and at confluence, and used to extract ATP. Cells were trypsinized, and an aliquot was used for the assay. An ATP determination kit (Invitrogen), based on the luciferin-luciferase assay, was used according to the manufacturer’s instructions. Measurements were made with a TD-20e luminometer (Turner Designs). The results are reported as nanomoles per 10⁶ cells.

**Mitochondrial membrane potential.** PMVECs and PAECs were grown to 60% confluence, and the media was replaced with Hank’s balanced salt solution containing JC-1 (5 μM), which is a cationic dye that denotes mitochondrial polarization. A change in fluorescence emission from green (525 nm) to red (590 nm) denotes low and high membrane potential, respectively. Cells were incubated at 37°C for 30 min, and then the dye was removed. Cells were examined with a fluorescence microscope, and pictures were taken with red and green filters. The pictures were analyzed using MetaMorph software.

**Inducible LDH-A knockdown.** Four pairs of short hairpin RNA (shRNA)-encoding oligonucleotides were designed using BLOCK-iT RNAi Designer program (Invitrogen), synthesized by Integrated DNA Technologies (Coralville, IA), reconstituted in water, annealed, and cloned into BsuL-digested pMA2867 (2). The resulting clones (pMA2894 1–4) were verified by sequencing and used to produce lentivirus-containing supernatants as described earlier (2). These supernatants were used to infect tetracycline-regulated (Tet-On) PMVEC (MV/2641). Infected cells were selected with puromycin (10 μg/ml).

**Statistics.** One- and two-way ANOVA and Student’s t-tests were used for statistical analyses, as appropriate. Bonferroni post hoc was used, as appropriate. Significance was denoted as P < 0.05.

**RESULTS**

PMVECs consume glucose and generate a lactic acidosis during growth. PMVEC populations grow faster than do PAEC populations (3, 6, 19, 30) due in large part to a high number of replication-competent cells within the monolayer (3). We confirmed these previous observations by performing population...
growth curves using both cell types (Fig. 1A). To measure cell growth, 10^5 cells were seeded onto 35-mm wells, and cell numbers were counted every day for 1 wk. PMVECs grew from 10^5 to >4 × 10^6 cells in 1 wk, whereas PAECs grew from 10^5 to 1 × 10^6 cells over this time course.

Media glucose (22 mM) was monitored throughout the growth curve. PMVECs consumed 76% of the total glucose over the 7-day growth curve; ~44% of the glucose was consumed during log-phase growth (e.g., days 3–5; Fig. 1B). In contrast, PAECs consumed just 20% of the total glucose during growth; ~4% of the glucose was consumed during log-phase growth. Glucose consumption was normalized to the cell number during log-phase growth. These data revealed that glucose depletion from the media was 3.4-fold higher in single PMVECs than in PAECs (5.7 × 10^{-5} mg·dl^{-1}·cell^{-1} vs. 1.7 × 10^{-5} mg·dl^{-1}·cell^{-1}). Estimates of cell volume suggest PMVECs and PAECs are similar in size (J. Creighton, unpublished observations).

Rapid glycolytic flux is commonly accompanied by acidosis. Increased glucose consumption in PMVECs was paralleled by an acidosis, as pH decreased from 7.5 on day 1 to 6.8 on day 7 of the growth curve, whereas media pH did not change in PAECs (Fig. 1C). Monocarboxylate transporters extrude protons with lactate across plasma membranes (9, 15, 20, 24), and therefore media lactate was measured. Throughout the growth curve time course, media lactate increased from 2 to 30 mmol/l in PMVECs and from 2 to 10 mmol/l in PAECs (Fig. 1D). Thus rapid PMVEC growth is accompanied by glucose consumption and lactic acidosis.

**Oxygen consumption is higher in PAECs than in PMVECs.** Oxygen consumption was measured in cell populations. Measurements were performed using 10^6 cells during log-phase (e.g., day 4) growth for both PMVECs and PAECs. PAECs (18 nmol-min^{-1}·10^6 cells^{-1}) consumed 2-fold more oxygen than did PMVECs (9 nmol-min^{-1}·10^6 cells^{-1}) (Fig. 2A). Consistent with these findings, mitochondria membrane potential was polarized in PAECs compared with PMVECs (Fig. 2B); quantification of these results revealed PAECs were 4.8 ± 0.8-fold more polarized than were PMVECs (n = 3 different studies, averaging ~20 cells per study, from 5 separate fields). Although glycolysis produces just 2 ATP molecules per molecule of glucose processed and is considered an inefficient mechanism of ATP synthesis compared with oxidative phosphorylation, rapid glycolytic flux can increase the ATP production in rapidly growing cells (16, 36). We therefore examined whole cell ATP concentrations in PMVECs and PAECs (Fig. 2C). During cell proliferation, ATP content was 2-fold higher in PMVECs than in it was in PAECs.

* mRNA and protein expression of key glycolytic enzymes in PMVECs and PAECs during growth.* Nine enzymatic reactions convert glucose to pyruvate, and in the case where pyruvate is not used for oxidative phosphorylation, 1 additional enzymatic step is required to convert pyruvate to lactate. Since glucose consumption and lactic acidosis coincided with rapid growth in PMVECs, we screened for expression of 3 key proteins regulating cellular glucose metabolism and lactate production, namely the GLUT-1, HK-2, and LDH-A. qRT-PCR and Western analysis revealed increased abundance of mRNA and protein in PMVECs in all 3 cases. Using qRT-PCR to analyze expression of GLUT-1, HK-2, and LDH-A, message was increased 4.5-fold (data not shown), 4.1-fold (data not shown), and 3.8-fold (data not shown), respectively, in PMVECs compared with PAECs. Since LDH-A activity is necessary to generate lactate, we further quantified LDH-A protein abundance. Similar to qRT-PCR results, LDH-A protein was nearly 7.5-fold increased in PMVECs compared with PAECs (Fig. 3).

**Glucose restriction limits PMVEC growth.** To test the degree to which PMVECs rely on glucose to sustain their rapid growth, cells were grown in three different glucose concentrations, including high (22 mM), intermediate (11 mM), and low (5 mM) levels; 11 mM glucose represents blood glucose concentrations in the CD40 rat. PMVEC growth was similar at high and intermediate glucose concentrations but was suppressed under low glucose conditions (Fig. 4A). Glucose was consumed in all experimental conditions. In studies using high glucose conditions, PMVECs reduced media glucose to ~5 mM, whereas in studies using intermediate and low glucose conditions, PMVECs consumed media glucose to negligible levels (Fig. 4B). The glucose consumption rates were similar under high and intermediate glucose conditions (5.7 × 10^{-5} and 5.4 × 10^{-5} mg·dl^{-1}·cell^{-1}, respectively) and reduced under low glucose conditions (3.1 × 10^{-5} mg·dl^{-1}·cell^{-1}; P < 0.05 vs. high and intermediate glucose concentrations). PMVEC growth in high and intermediate glucose media resulted in lactic acidosis (Fig. 4, C and D), as noted in Fig. 1. In contrast, under low glucose conditions, cell growth was not accompanied by lactic acidosis. Notably, the PMVEC growth capacity in low glucose concentrations was similar to the PAEC growth capacity in high glucose concentrations (Fig. 1).

We determined whether glucose restriction similarly inhibited PAEC growth. In stark contrast to the results in PMVECs, reducing media glucose did not diminish the PAEC growth capacity (Fig. 5A). Glucose was only nominally consumed at all concentrations (Fig. 5B), and neither pH (Fig. 5C) nor lactate concentration (Fig. 5D) were altered by media glucose in the macrovascular cells.

![Fig. 3. Lactate dehydrogenase A (LDH-A) was upregulated in PMVECs compared with PAECs. Whole cell lysates were collected during log-phase growth, and Western blotting was performed to assess protein abundance. LDH-A abundance was increased in PMVECs compared with PAECs. Unpaired 2-tailed t-test was used to assess significance. *P < 0.05 in PMVECs vs. PAECs with n = 4.](http://ajplung.physiology.org/)
Galactose inhibits PMVEC growth, an effect partially rescued by exogenous lactate. If PMVECs consume glucose to sustain rapid growth, then inhibiting glycolysis should reduce their growth rate. To address this issue, extracellular glucose was replaced with galactose (22 mM), which represents an inefficient substrate for glycolysis. PMVECs grown in galactose increased in cell number from 10^5 to 2 × 10^6 cells over a 7-day growth curve (Fig. 6A), achieving a peak cell number that resembled PAECs grown in high glucose (Fig. 1A) and PMVECs grown in low glucose (Fig. 4A). Lactate did not accumulate in the media of cells grown in galactose-containing media (data not shown). To determine whether growth could be rescued by replenishing glucose, galactose-containing media was replaced with glucose-containing media on day 4 of the growth curve. Addition of extracellular glucose rescued PMVEC growth to near its usual capacity, 4 × 10^6 cells.

Since lactate accumulates in the media of cells grown using glucose media, we investigated whether lactate possesses an independent role in controlling cell growth. Lactate was measured in the buffer of PMVECs grown with high glucose concentrations. Using these known lactate concentrations, exogenous lactate was added to the buffer of PMVECs grown in galactose-containing media (Fig. 6B). Replenishing lactate to cells grown in galactose-containing media partially rescued cell growth. Moreover, whereas total cellular ATP concentrations were reduced in PMVECs grown in galactose-containing media, replenishing the galactose-containing media with lactate restored ATP concentrations to normal levels, suggesting lactate was converted into pyruvate, and pyruvate entered the mitochondria for aerobic respiration (Fig. 6C).

LDH-A activity is necessary to sustain rapid proliferation in PMVECs. LDH converts pyruvate to lactate, which provides NAD^+ needed for enzymatic reactions in glycolysis. We examined whether LDH activity is necessary to sustain PMVEC growth using a pharmacological inhibitor, the pyruvate analog oxamate (21, 29). Oxamate dose-dependently (4–16 mM) decreased lactic acidosis and cell growth (data not shown). At the highest oxamate concentration tested (16 mM), inhibition of both lactic acidosis and cell growth was similar to that seen in galactose-treated cells.

To specifically test whether LDH-A is necessary to sustain PMVEC growth, four shRNA probes were generated and tested; one significantly reduced LDH-A expression and was chosen for further study (data not shown). PMVECs were engineered for Tet-On conditional expression of LDH-A shRNA (2) (Fig. 7, A and B). Doxycycline dose-dependently decreased LDH-A expression (data not shown); optimal LDH-A inhibition was observed at low doxycycline (~3 μg/ml) concentrations. LDH-A suppression was achieved for an extended time period by replenishing the media daily with doxycycline, as the doxycycline half-life is 24 h (Fig. 7C). Using this conditional expression approach, doxycycline in-
duced shRNA that suppressed LDH-A for 7 days without decreasing expression of LDH-B (data not shown). Whereas the uninduced cells grew to $5 \times 10^6$ cells in a standard growth curve, as is typical for wild-type PMVECs (Fig. 1A), doxycycline treatment decreased PMVEC growth to approximately $2 \times 10^6$ cells (Fig. 7D), similar to that seen in wild-type PAECs (Fig. 1A), suggesting LDH-A critically contributes to PMVEC proliferation. Suppressing the expression of LDH-A decreased glucose consumption (Fig. 7E) and lactate production (Fig. 7F) throughout the growth curve.

To test whether growth could be rescued, doxycycline was added to cells for 3 consecutive days and then withdrawn for 7 days. Doxycycline treatment decreased LDH-A expression, and on withdrawal, LDH-A expression recovered (Fig. 7G). Whereas PMVEC growth was suppressed in the continued presence of doxycycline, on doxycycline withdrawal, PMVEC growth returned to normal levels, $5 \times 10^6$ cells (Fig. 7H).

**DISCUSSION**

Highly proliferative cells display aerobic glycolysis, which is characterized by rapid glycolytic flux, preferential conversion of glucose into lactate, and a corresponding lactic acidosis (16, 36). This atypical metabolic profile was first recognized in Warburg’s studies of cancer cell metabolism and became known as the Warburg effect (39, 40). Four lines of evidence support the idea that PMVECs exhibit aerobic glycolysis. First, rapidly growing PMVECs consumed media glucose throughout their growth from single cells into a confluent monolayer. Either restricting glucose uptake by lowering media glucose or replacing glucose with galactose blunted the rapid proliferation observed in PMVECs. Second, glucose consumption was paralleled by development of a lactic acidosis. PMVECs, but not PAECs, generated a lactic acidosis that required glucose consumption. Whereas ATP concentrations were higher in PMVECs than they were in PAECs, oxygen consumption was lower in the microvascular cells. Third, proteins responsible for glucose uptake and fermentation of glucose to lactate, namely GLUT-1, HK-2, and LDH-A, were all increased in PMVECs compared with PAECs. Fourth, high glucose consumption, generation of lactic acidosis, and upregulation of GLUT-1, HK-2, and LDH-A occurred under normoxic conditions. Indeed, the metabolic profile displayed by PMVECs represents the observations first made by Warburg (39, 40).

LDH converts pyruvate into lactate and in so doing generates NAD⁺ important to sustain glycolytic flux. The functional LDH enzyme is a homodimer or heterodimer comprising different -A and -B subunit combinations. Expression of LDH-A is characteristic of rapidly growing cells, as LDH-A is upregulated in rapidly growing cells, and suppressing LDH-A expression stimulates mitochondrial respiration and decreases growth (10). In cancer cells, inhibiting LDH-A mitigates their tumorigenicity (10). Our results support this general idea, as...
rapidly growing PMVECs expressed a greater abundance of LDH-A than did PAECs, and inhibiting LDH-A reduced growth.

It is not presently clear why PMVECs acquire this metabolic function, although it likely reflects the unique angioproliferative demands of the microcirculation. PMVECs are derived from the microcirculation of the lung, whereas PAECs are obtained from conduit pulmonary arteries (3, 6, 12, 19, 23, 31). Both cell types express accepted endothelial markers, including endothelial cell nitric oxide synthase and vascular endothelial cell adhesion molecule. Both cell types are capable of forming vascular networks on Matrigel in vitro and blood vessels in Matrigel plugs in vivo, although PMVECs display greater angioproliferative capacity than do the macrovascular cells. PMVECs and PAECs differ in their ability to interact with lectins. PMVECs interact with *Griffonia simplicifolia* in vivo and in vitro, whereas PAECs do not; PAECs interact with *Helix pomatia* in vivo and in vitro, whereas PMVECs do not (3, 6, 12, 19). PMVECs also differ from PAECs in the number of replication-competent cells within their populations (3). Single cell cloning experiments reveal that 40–50% of PMVECs are highly proliferative, whereas only 3–5% of PAECs are highly proliferative. *G. simplicifolia* binding does not indicate a highly proliferative cell, as nondividing single cells obtained from PMVEC populations interact with this lectin, and single cells obtained from PAEC populations that are replication-competent do not interact with *G. simplicifolia*. The rapid growth of PMVECs appears to represent an intrinsic feature of the microvascular cells.

Nitric oxide is a key determinant of endothelial cell bioenergetics (7, 26). Nitric oxide competes with oxygen for binding to cytochrome c oxidase and in so doing inhibits mitochondrial respiration. Indeed, this action of nitric oxide in endothelium likely contributes to the relatively low level of mitochondrial respiration in these cells and to their reliance on glycolysis for ATP production (7, 26). Although PMVECs and PAECs both express endothelial cell nitric oxide synthase (type III NOS), PMVECs possess less of this enzyme and produce less nitric oxide than do PAECs (A.-B. Al-Mehdi, unpublished observations). Our results using PMVECs are consistent with those from Xu and colleagues (43) in which rapidly proliferating PAECs isolated from patients with idiopathic pulmonary arterial hypertension were shown to possess decreased basal nitric oxide levels. However, PMVECs possessed reduced rates of oxygen consumption compared with PAECs and consequently were more reliant on glycolysis. It would therefore appear that whereas endothelial cells use the autocrine production of nitric oxide to shift metabolism toward glycolysis, PMVECs use other mechanisms to account for their reliance on glycolytic flux to support rapid proliferation.

**Fig. 6.** Exogenous lactate partially rescues PMVEC growth in glucose-deficient media. A: substitution of glucose for galactose (22 mM) inhibited PMVEC growth. Adding glucose at day 4 of the growth curve rescued PMVEC growth. Arrow denotes the time at which glucose was added to the cells grown in galactose. Supplying extracellular lactate to PMVECs grown in galactose-containing media promoted PMVEC growth (B) and increased ATP levels (C). One-way ANOVA was used to assess significance over the 7-day time course, 2-way ANOVA was used to compare between treatments, and Bonferroni post hoc test was performed as needed. *Significantly different (P < 0.05; n = 4). ^Significantly different (P < 0.05) from baseline at day 1. Unpaired t-test was used to compare ATP concentrations among galactose treatment (P < 0.05; n = 3).
Fig. 7. LDH-A is required for PMVECs to sustain aerobic glycolysis and rapid growth. 

**A**: PMVECs were infected with a retrovirus (cat. no. 2641), enabling reverse tetracycline-controlled transactivator protein (rtTA) expression. Cells were selected to homogeneity using blasticidin, reinfected with a lentivirus (cat. no. 2894-2), and selected to homogeneity using puromycin. The resulting double-transfection enabled doxycycline-responsive expression of a LDH-A short hairpin RNA (shRNA). Bsr, blasticidin resistance gene; EGFP, enhanced green fluorescent protein; HIV RRE, human immunodeficiency virus Rev response element; IRES EMV, encephalomyocarditis virus internal ribosome entry site; LTR, retro/lentiviral long terminal repeat; PAC, puromycin resistance gene; PSV40, simian virus 40 promoter; PTet, doxycycline-regulated promoter; wPRE, woodchuck hepatitis virus posttranscriptional regulatory element; mir, 5′–3′ flanking sequence derived from the murine mir (micro-RNA gene)-15. 

**B**: doxycycline promoted expression of the red fluorescent protein mCherry in PMVECs infected with both retrovirus cat. no. 2641 and lentivirus cat. no. 2894-2 and selected to homogeneity using blasticidin and then puromycin. mCherry fluorescence reveals the uniform doxycycline-responsive shRNA expression. 

**C**: Western analysis demonstrates that daily doxycycline treatment decreases LDH-A protein over a 7-day time course, resulting in decreased PMVEC growth (D), glucose consumption (E), and lactate production (F). Whereas doxycycline treatment decreases LDH-A protein (G) and PMVEC growth (H), both LDH-A protein and PMVEC growth are rescued on doxycycline withdrawal. One-way ANOVA was used to assess significance over the 7- or 10-day time course. 2-way ANOVA was used to compare between groups, and Bonferroni post hoc test was performed as needed. *Significantly different (P < 0.05; n = 3). ^Significantly different (P < 0.05) from baseline at day 1.
Nucleosome assembly protein-1 expression is a key determinant of endothelial cell proliferation (6). Rapidly growing cells express high levels of nucleosome assembly protein-1. Inhibiting nucleosome assembly protein-1 expression decreases growth in rapidly dividing cells, and heterologous expression of nucleosome assembly protein-1 converts slow-growing cells into rapidly dividing cells. Nucleosome assembly protein-1 may therefore represent an epigenetic factor fundamentally important in establishing the Warburg-like phenotype observed in PMVECs. In addition, glucose consumption in rapidly growing cells is necessary to produce acetyl-coenzyme A (27, 41, 42). ATP citrate lyase uses acetyl-coenzyme A as a substrate for histone acetylation. Work from Wellen and colleagues (42) recently demonstrated that such glucose-dependent histone acetylation underlies the upregulation of glycolytic enzyme intermediates such as HK-2 and LDH-A. It will be important to determine how the functions of nucleosome assembly protein-1 and ATP citrate lyase, independently or collectively, regulate the proliferative phenotype of endothelium.

It is anticipated that the aerobic glycolysis described presently represents an important mechanism by which PMVECs sustain rapid angiogenesis, important for vascular repair. This idea is not unprecedented, as aerobic glycolysis has previously been incriminated in wound healing, where rapid tissue repair is essential (35). Lactate production appears to be a critical determinant of wound healing (35). Whereas traditional thinking considered lactate as merely a metabolic by-product, recent findings challenge this idea. Aerobic glycolysis may be necessary for the fatty acid, lipid, nucleic acid, and amino acid synthesis that is concomitantly needed to support growth (36). LDH-A inhibition prevents lactate accumulation and the corresponding rapid growth of tumor cells (10). These findings indicate that aerobic glycolysis may not only be relevant to support ATP synthesis, but also to support the carbon biomass synthesis that is needed for rapid growth. We observed that when PMVECs were grown in galactose-containing media rather than in glucose-containing media, lactate production was abolished, and growth was impaired. Restoring lactate was sufficient to fully restore cellular ATP concentrations and partially rescue cell growth. These findings suggest that lactate fulfills an independent signaling role in control of cell growth. How lactate accomplishes this metabolic benefit in PMVECs is currently unclear, although lactate may act as a pseudohypoxia (35) or pseudohormonal (25) signal or may contribute to the lactate shuttle.

In summary, we report that PMVECs display aerobic glycolysis during their growth, including glucose consumption and lactic acidosis, which is dependent on the expression of LDH-A. This distinctive metabolic behavior is necessary for them to sustain rapid growth, as limiting glucose or replacing glucose abolishes the lactic acidosis and prevents rapid proliferation. Lactate plays a key role in sustaining PMVEC growth, as replenishing lactate even in the absence of extracellular glucose is sufficient to restore ATP levels and partially rescue cell growth. Future studies will be required to determine the molecular mechanisms responsible for imprinting this proliferative phenotype in PMVECs.

ACKNOWLEDGMENTS

We thank Drs. Brian Fouty and Mark Gillespie for their helpful discussions, Anna Budford and Linn Ayers for assistance in cell culture, and Dr. Chris Morris for assistance with statistical analyses.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-60024 and HL-66299 (to T. Stevens).

DISCLOSURES

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

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