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Mitochondrial and Metabolic Drivers of Pulmonary Vascular Endothelial Dysfunction in Pulmonary Hypertension

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1 Introduction

The pulmonary vascular endothelium comprises a single layer of endothelial cells (ECs) on the inner layer of the vessel wall and controls a variety of vessel functions. These ECs participate in the regulation of vascular tone and barrier, leukocyte trafficking, blood coagulation, nutrient and electrolyte uptake, and neovascularization of hypoxic tissue [1]. Pulmonary hypertension (PH) describes a heterogeneous and deadly set of vascular disease conditions, defined by increased pulmonary arterial pressure and lung vasculopathy, triggered by varied and often disparate stimuli [2]. Although PH has been a historically neglected disease, its significance and documented prevalence worldwide are growing due to improvement in awareness and diagnostic capabilities for detection [3]. Current treatment modalities of PH focus primarily on vasomotor tone and limited efficacy or specificity on other cellular processes [4]. At the molecular level, while many cell types within and outside the pulmonary arterial wall are crucial to PH development, endothelial dysfunction is thought to be a major contributor to overall pathogenesis. Yet endothelial dysfunction in PH remains incompletely defined and is thought to be marked by both altered apoptosis and proliferation via a precisely regulated spatiotemporal molecular orchestration [5]. These processes are tightly linked to the development of oxidative stress, inflammatory response, and adverse remodeling of the pulmonary vasculature and are recognized as some of the initiating events of PH under various genetic and exogenous stresses [2]. Importantly, alterations in endothelial mitochondrial and metabolic functions in the pulmonary vasculature are emerging as prominent regulators of endothelial dysfunction and consequently of PH pathogenesis. In contrast to numerous cell types, endothelium has historically been thought to function relatively independent of the mitochondrial pathway of energy supply, and synthesis of ATP in endothelium occurs mainly via a glycolytic pathway [6]. However, as evident from recent studies, mitochondrial modulation of free radicals, calcium homeostasis, and iron–sulfur clusters in endothelial cells can control responses to inflammation, oxidative stress, and apoptotic stimulus. This chapter reviews the roles of

mitochondria in pulmonary ECs, with a particular focus on how these organelles can modulate endothelial dysfunction in PH. We propose that the study and in-depth understanding of pulmonary EC metabolism may offer powerful therapeutic targets for the next generation of drugs designed to reverse or prevent PH.

2 Mitochondria in Endothelial Metabolism and Energy Reserve

Mitochondria are essential to cellular energy production in all higher organisms adapted to an oxygen-containing environment. Yet the endothelium is not considered to be a major energy-requiring tissue. In comparison with other cell types with higher energy requirements, mitochondria content in ECs is modest at best. In rat ECs, for example, mitochondria compose 2–6% of the cell volume as opposed to 28% in hepatocytes and 32% in cardiac myocytes [7, 8]. Despite their close proximity to oxygenated blood, ECs rely mainly on anaerobic glycolysis instead of mitochondrial oxidative metabolism for adenosine triphosphate (ATP) production. In fact, under physiological conditions, ECs produce over 75% of their ATP from the conversion of glucose to lactate. Less than 1% of glucose-derived pyruvate enters the tricarboxylic acid cycle (TCA) and hence the low mitochondrial electron transport chain (ETC) respiration dedicated for oxidative metabolism [9]. Because ECs rely much more on glycolytic metabolism than other cell types, the potential pathophysiological role of their mitochondria has been, to some degree, neglected. However, ECs retain an extensive mitochondrial network and the ability to switch to oxidative metabolism of glucose, amino acids and fatty acids in case of reduced glycolytic rates [10]. For example, bovine aortic ECs have been shown to use only approximately 35% of their maximal oxygen consumption at basal conditions, and mitochondria in these endothelial cells are highly coupled and possess a considerable bioenergetic reserve. The presence of a reserved respiratory capacity that is available for ECs when bioenergetic demand is increased suggests that mitochondria may function as stress sensors and signaling initiators in ECs [11].

3 Repression of Endothelial Oxidative Phosphorylation in PH

In contrast to the baseline state, the diseased state of the endothelium highlights the importance of mitochondrial metabolism to overall cellular (patho)phenotype. EC dysfunction and apoptosis appear to be an early event in PH. In PH, a widely cited theory exists that initial EC apoptosis gives rise to a separate population of hyperproliferative and pathogenic ECs that drive later stages of disease. This was originally described by Voelkel and colleagues [12] in 2005, which has been echoed by others since then [5, 13]. Interestingly, the hyperproliferative and apoptosis-resistant ECs exhibit a metabolic profile strikingly similar to that of cancer cells [7]. Cancer cells show reliance upon glycolysis and shift away from oxidative phosphorylation for cellular energy production, a phenomenon known as the Warburg effect [14]. Yet, aerobic glycolysis is not just a unique feature of malignancy; it is also observed in nontransformed proliferating cells when glucose is sufficient [15].

Studies in human, rodent, avian and lamb PH have demonstrated that ECs in PH also produce energy primarily from glycolytic metabolism [16–19], which is analogous to the

alterations in cancer cell metabolism [20]. In normal ECs, ATP is generated nearly equivalently by glycolysis and mitochondrial respiration [9], representing a relatively high glycolytic activity compared to other cell types and increased tolerance to hypoxia because of low oxygen demand. Indeed, cellular ATP content of ECs of PH models is similar to control cells under normoxia; however, under hypoxia, cellular ATP remains static in PH endothelial cells but decreases significantly in control endothelial cells [21]. The greater tolerance to hypoxia suggests a predominant anaerobic metabolism in PH ECs and a lesser dependence upon mitochondrial respiration.

A number of metabolic control points have been identified in the repression of endothelial oxidative phosphorylation observed in PH. Overall, as we describe in Fig. 1, these include: (1) activation of hypoxia-induced factor-1 (HIF-1), which upregulates pyruvate dehydrogenase kinase (PDK), hence suppressing mitochondrial oxidative phosphorylation [22]; (2) diversion of pyruvate into anabolic pathways [23]; (3) inhibition of voltage-gated K⁺ channels (Kv channels), thereby increasing cytosolic Ca²⁺, inducing the hyperproliferative transcription factor nuclear factor of activated T cells (NFAT), and activating glycolytic enzymes [24]; and (4) suppression of apoptosis by hyperpolarized mitochondrial membrane potential (Ψ_m) [25].

Notably, it has been speculated that decreased mitochondrial function may be a primary stimulus to glycolysis [26]. There is evidence supporting intrinsic deficiencies in mitochondrial function in the metabolic shift to glycolysis in PH [16, 19] as well as secondary mitochondrial changes following endothelial dysfunction. Site-specific defects in the ETC within complexes I and III have been identified in avian spontaneous idiopathic PH, where the lower respiratory chain coupling, inefficient use of oxygen, and increased ROS generation are directly related to the development of PH [17]. Similarly, fawn-hooded rats, a spontaneously PH rodent strain, present dysmorphic mitochondria with reduced expression of ETC components (complexes I, III, and IX), reduced expression of manganese superoxide dismutase (MnSOD), depressed mitochondrial reactive oxygen species (ROS) production, and activated PDK which shift metabolism away from oxidative phosphorylation toward glycolysis. The abnormalities in mitochondrial function have been found to activate the master transcription factor of hypoxia, HIF-1 α , which then can inhibit expression of oxygen-sensitive Kv channels, analogous to the pathophysiology of chronic hypoxic exposure [16]. Strikingly, dichloroacetate (DCA), a mitochondrial PDK inhibitor, was found to reverse PH in fawn-hooded rats, providing a mechanistic relationship between mitochondrial function and PH pathogenesis [18]. The decreased expression and activity of mitochondrial enzymes required for carnitine metabolism in lambs also lead to mitochondrial dysfunction, via decreased MnSOD expression and increased uncoupling protein-2 expression, thus increasing glycolysis, endothelial dysfunction, and ultimately PH [19]. Similarly, pulmonary artery endothelial cells from human idiopathic PH lungs have revealed decreased mitochondrial dehydrogenase activity, mitochondrial number and mitochondrial DNA content per cell as well as higher glycolytic rate than in control cells [27]. In vitro study of human PAH ECs demonstrated that they have lower than normal oxygen consumption for state 3 and state 4 respiration with glutamate-malate or succinate as substrate [27]. It was also observed that activity of complex IV, the terminal enzyme complex of the respiratory chain that catalyzes the transfer of electrons from reduced

cytochrome *c* to molecular oxygen, is significantly lower, while complex III activity is similar among control and PH ECs. Notably, the coupling between oxygen consumption and ATP production, the respiratory control index of mitochondrial function, was also found to be similar between PH and control cells. Thus, human PH ECs appear to display lower numbers of active mitochondria, which result in greater reliance upon glycolysis [13].

4 Balancing Endothelial Proliferation and Glycolysis in PH: The Role of Anaplerosis

Glycolysis is likely advantageous to rapidly growing cells, as it renders them less dependent on oxygen, thereby improving cell survival in an environment that may become hypoxic as cell number increases. Furthermore, the transition to glycolysis in proliferating cells minimizes exposure to reactive oxygen species (ROS) [28]. Yet glycolysis is far less effective in meeting the enhanced ATP demand of proliferating cells. Indeed, increased pulmonary arterial EC (PAEC) proliferation has been noted by multiple groups and linked to PH pathogenesis. Historically, this has included the study of plexiform lesions (previously described as “disorganized endothelial proliferation” [29]) which are pathognomonic signs of this disease. Moreover, recent studies such as Kim et al. [30] have demonstrated that cultured PAECs isolated from human PAH patients display increased proliferation compared with non-diseased control cells. Other studies have isolated highly proliferative endothelial cells from PAH patients as well [31]. Studies in other animal models of PH (to name a few) such as transgenic mice expressing interleukin-6 [32], long-term inhibition of the potassium channel KCNK3 (Potassium Two Pore Domain Channel Subfamily K Member 3) in rats [33], and monocrotaline-exposed rats [30] all display increased PAEC proliferation in diseased arterioles. However, in PH as in cancer, the metabolic needs of rapid proliferation cannot be met by glycolysis alone.

In that context, even in the setting of the glycolytic state, the TCA cycle can serve as a primary source of energy production via the replenishment of carbon intermediates. This process known as anaplerosis is accomplished via two major pathways: glutaminolysis (deamidation of glutamine via the enzyme glutaminase [GLS1]) and carboxylation of pyruvate to oxaloacetate via ATP-dependent pyruvate carboxylase. Specifically, glutaminolysis via GLS1 activity contributes to anaplerosis by allowing for mobilization of cellular energy, carbon, and nitrogen, particularly in rapidly proliferating cells [34] and serves as a crucial process in transformed cells that have switched their metabolism from oxidative phosphorylation to glycolysis in order to maintain cell growth and viability [35]. Recently, our group reported a novel link between vascular stiffness and EC glutaminolysis that drives the proliferative vascular phenotype in PH [36]. Namely, in cultured pulmonary vascular cells and in PH samples, including primate and human instances of human immunodeficiency virus-induced PH, we found that ECM stiffening mechanoactivated the transcriptional co-activators YAP (Yes-associated protein 1) and TAZ (Transcriptional activator with PDZ binding motif 1) to modulate metabolic enzymes including GLS1 and thus coordinate glutaminolysis and glycolysis (Fig. 2). Glutaminolysis replenished aspartate for anabolic biosynthesis (anaplerosis), sustaining proliferation and migration within stiff extracellular matrix. In vivo, pharmacologic modulation of pulmonary vascular stiffness and

YAP-dependent mechanotransduction altered glutaminolysis, pulmonary vascular proliferation, and manifestations of PH. Correspondingly, using two separate GLS1 inhibitors, pharmacologic targeting of GLS1 ameliorated PH progression. Thus, our data demonstrate that ECM stiffening sustains vascular cell growth and migration through YAP/TAZ-dependent glutaminolysis and anaplerosis—a paradigm that advances our understanding of the connections of mechanical stimuli to dysregulated endothelial metabolism. These results also indicated the possibility of novel glutaminolytic targets in ECs for the treatment of PH. Additional triggers of this EC metabolic shift likely exist and potentially are regulated by both protein factors as well as other noncanonical effectors (i.e., microR-NAs [37]), but these await further investigation.

5 Endothelial Mitochondria as Stress Sensors in PH

Mitochondria critically determine cell survival and death by regulating ATP synthesis. Besides oxidative ATP production, however, mitochondria hold other essential regulatory roles in heme synthesis, β -oxidation of free fatty acids, metabolism of certain amino acids, production of free radical species, formation and export of iron–sulfur clusters, iron metabolism, and calcium homeostasis [6]. Specific mitochondrial abnormalities related to clinical PH and experimental PH include induction of a pseudohypoxic state via activation of HIF1 α , impaired mitochondrial–ER interaction, and mitochondrial fission [38]. Dysmorphic mitochondria with defects in the ETC, decreased respiratory chain coupling, and inefficient use of oxygen have also been reported [27]. A recent study revealed that mice with endothelial deletion of bone morphogenetic protein receptor 2 (BMPR2) develop PH following hypoxia-reoxygenation by increasing mitochondrial ROS production, reducing mitochondrial membrane potential, and inhibiting mitochondrial biogenesis [39]. These findings suggested a critical role of endothelial mitochondria in the pathogenesis of BMPR2 deficiency—a heterozygous genetic state found in approximately 70% of familial PAH and in 20% of sporadic cases of idiopathic PAH [40]. Another example is the connection between dasatinib-induced PH and endothelial mitochondrial dysfunction. Dasatinib, a dual Src and BCR-ABL tyrosine kinase inhibitor used to treat chronic myelogenous leukemia, causes pulmonary vascular damage by inducing ER stress and mitochondrial ROS production in the endothelial cells, which leads to increased susceptibility to PH development [41].

5.1 Mitochondrial ROS in Pulmonary ECs

Beyond the production of ATP, the generation of ROS as secondary messengers allows endothelial mitochondria to act as a primary signaling organelle in the cell. In the hypoxic lung, mitochondrial ROS production has been localized predominantly to complex II [42], although complexes I and III are recognized to produce most of the ROS in other occasions. In a monocrotaline-induced rat model of PH, selective increases in the expression and activity of complex II were observed as well as complex II-derived ROS [43]. Exposure of PAECs to hypoxia triggers the perinuclear clustering of mitochondria and accumulation of ROS in the nucleus, which can be attenuated by nocodazole to destabilize microtubules and inhibit retro-grade mitochondrial movement [44]. Evidence also supports the critical roles of increased mitochondrial ROS production and reduced MnSOD activity in the pathogenesis

of PH, events that also contribute, via consumptive reactions, to the loss of the vasodilator nitric oxide (NO) often observed in PH. For example, mitochondrial derived ROS have been shown to induce pulmonary vascular remodeling in PH by increasing intracellular Ca^{2+} , inducing Ψ_m depolarization/hyperpolarization, and PAEC apoptosis and pulmonary artery smooth muscle cell proliferation [45]. The increase in mitochondrial ROS in PH has been linked to elevated circulating levels of asymmetric dimethylarginine (ADMA), which induces mitochondrial dysfunction by increasing uncoupling protein-2 (UCP-2) protein levels and reduction in cellular ATP levels [46]. Furthermore, supplementation of ECs with mitochondria-targeted antioxidants inhibits peroxide-induced mitochondrial iron uptake, oxidative damage, and apoptosis [47]. Antioxidants such as dimethylthiourea [48], recombinant human SOD [49] and *N*-acetyl-L-cysteine [50], a precursor to the nonenzymatic antioxidant glutathione, have all been shown to significantly abrogate the pulmonary hypertensive response and consequent right ventricular dysfunction. However, reduced mitochondrial ROS have also been found in hypoxia and monocrotaline-induced PH, as well as in the fawn-hooded rats [16, 51]. Other studies have also demonstrated that hypoxia attenuates ROS production in the rabbit lung [52], and inhibition of the mitochondrial ETC with rotenone and antimycin A results in reduced ROS production and pulmonary vasoconstriction [53]. These discrepancies may be due to the inherent differences in the models employed or the progression of the disease. Thus, the role of mitochondria-derived ROS in PH is complex, and much work lies ahead to clarify these complex issues.

5.2 Mitochondrial Ca^{2+} in Pulmonary ECs

Agonist stimulated increase in cytosolic Ca^{2+} in endothelial cells has been repeatedly linked to accumulation of Ca^{2+} in the mitochondria [54]. This is achieved by voltage-dependent anion channel (VDAC) in the outer mitochondrial membranes and mitochondrial Ca^{2+} uniporter (MCU) in the inner mitochondrial membranes [55]. Mitochondrial Ca^{2+} and its buffering of the cytosolic Ca^{2+} serve as important orchestrators of mitochondrial biogenesis and many downstream aspects of mitochondrial functions including mitochondrial ROS production, aerobic metabolism, dynamics and biogenesis [8]. Such processes cooperate with the endoplasmic reticulum (ER) to maintain cellular Ca^{2+} homeostasis. Indeed, the distance between the ER and mitochondria has been associated with intramitochondrial Ca^{2+} , mitochondrial membrane potential (Ψ_m) and mitochondria-dependent apoptosis in pulmonary arterial cells in PH [56]. The accumulation of Ca^{2+} in the mitochondrial matrix ($[\text{Ca}^{2+}]_{\text{mt}}$) leads to the activation of several Ca^{2+} -sensitive effectors, including key metabolic enzymes such as pyruvate dehydrogenase phosphate phosphatase and NAD^+ -isocitrate dehydrogenase [57], as well as mitochondrial nitric oxide synthase, thus producing nitric oxide which relaxes smooth muscle cells by increasing cytosolic cGMP [58]. Additional work will be necessary to determine whether pharmacologic modulation of calcium handling specifically in PAECs independent of smooth muscle cells may be effective for more precise endothelial therapies in PH.

5.3 Mitochondrial Iron–Sulfur (Fe–S) Clusters in Pulmonary ECs

Recently, the importance of iron–sulfur (Fe–S) clusters to endothelial metabolism in PH has been described. Fe–S clusters ($[4\text{Fe-4S}]$ and $[2\text{Fe-2S}]$) are critical bioinorganic prosthetic groups that promote electron transport and oxidation–reduction reactions integral to

numerous cellular processes ranging from ribosome biogenesis, purine catabolism, heme biosynthesis, DNA repair, and iron metabolism, among others [59]. In particular, [Fe-S] clusters are essential components of enzymes involved in the maturation of subunits of complexes I, II, and III, which facilitate electron transport. Fe-S clusters also participate in the synthesis of the enzyme-bound cofactor lipoate present in the 2-oxoacid dehydrogenases including pyruvate dehydrogenase and oxoglutarate dehydrogenase complexes, which are integral to TCA cycle function [59]. Depending upon the level of ambient oxygen exposure, alteration of these and other iron-sulfur-dependent mitochondrial activities can lead to distinct downstream consequences on ROS production and cellular survival [60]. Despite their established importance in these cellular redox reactions, regulation and function of these critical prosthetic groups in hypoxic mammalian cells are poorly characterized. The formation of Fe-S clusters is controlled by a conserved set of assembly and scaffold proteins. Current knowledge regarding the importance of these proteins in human disease has been derived largely through investigation of genetic mutations [61]. For example, mutations in Fe-S cluster scaffold genes NFU1 and BOLA3 have been linked to the multiple mitochondrial dysfunction syndrome (MMDS), a fatal and rare autosomal recessive disease characterized by deficiency of complexes I, II, and III in the mitochondrial respiratory chain and 2-oxoacid dehydrogenase enzymes [62]. Notably, histologic analysis of deceased infants carrying NFU1-mutations and suffering from MMDS has revealed substantial pulmonary vascular remodeling [63]. Correspondingly, in PAECs from PH mice, downregulation of a separate Fe-S biogenesis gene, ISCU, in PAECs was observed, compromising integrity of Fe-S clusters [64], repressing mitochondrial oxidative phosphorylation, increasing ROS, and promoting PH in mice. Importantly, cardiopulmonary exercise testing of a woman with homozygous ISCU mutations also has revealed exercise-induced pulmonary vascular dysfunction [65]. Thus, Fe-S deficiency may represent an overarching point of regulation promoting PH via metabolic dysregulation, and it remains to be seen whether other Fe-S biogenesis genes can similarly influence PH inception or progression.

6 Conclusions

In summary, it is increasingly clear that dysregulated endothelial metabolism and mitochondrial function drive primary aspects of PH from inception to end-stage disease. Yet the molecular details of this activity are not fully defined. Currently, it is thought that endothelial mitochondria may participate in PH through two main mechanisms: (1) alterations of glycolysis, oxidative phosphorylation, and anaplerosis to provide substrates for proliferation and (2) generation of secondary signaling messengers (i.e., ROS, Ca^{2+}) to promote downstream endothelial pathophenotypes. Future work will entail further definition of the molecular triggers and regulatory checkpoints of these processes (Fig. 3). In doing so, it is hoped that these discoveries could be translated rapidly into clinical applications for introducing a new era of metabolic diagnostics and therapeutics for PH and its devastating consequences.

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Fig. 1.

Relationships between metabolome, proteome, and genome in metabolic reprogramming of endothelial cells in PH. In typical cells under normal oxygen levels, a majority of pyruvate derived from glucose enters the mitochondria where it is oxidized in the TCA cycle to generate ATP to meet the cell's energy demands. However, in PH endothelial cells, pyruvate is directed away from the mitochondria toward glycolysis in order to create lactate through the action of lactate dehydrogenase (LDH)—a process typically activated by low oxygen exposure. Lactate production in the presence of oxygen is termed “aerobic glycolysis” or the Warburg effect, a phenomenon common in cancer cells. In aerobic glycolysis, excess glucose is diverted through the pentose phosphate shunt (PPS) and serine/glycine biosynthesis pathway to create nucleotides. Fatty acids are critical for new membrane production and are synthesized from citrate in the cytosol by ATP-citrate lyase (ACL) to generate acetyl-CoA. Signals impacting levels of hypoxia inducible factor (HIF) and nuclear factor of activated T cells (NFAT) can increase expression of enzymes such as LDH to promote lactate production, as well as pyruvate dehydrogenase kinase to inhibit the action of pyruvate dehydrogenase and limit entry of pyruvate into TCA cycle. Highly proliferative endothelial cells need to produce excess lipid, nucleotide, and amino acids for the creation of new biomass. There is also increased use of glutamine as another fuel source, which enters the mitochondria and can be used to replenish TCA intermediates or to produce more pyruvate through the action of malic enzyme. Adapted with permission from *Science* [66]

Fig. 2.

Pulmonary vascular stiffness controls major metabolites in anaplerosis and glycolysis in cultured pulmonary vascular cells and in PH-diseased primate and human samples. Glutaminase (GLS1) and pyruvate carboxylase (PC) generate the major anaplerotic metabolites (*blue*), feeding into the TCA cycle (*black*) and supporting the anabolic demand for biosynthesis (*green*). Lactate dehydrogenase A (LDHA) modulates glycolysis (*red*). As compared with soft ECM (*left panel*), ECM stiffening (stiff ECM, *right panel*) mechanoactivates the transcriptional co-activators YAP/TAZ to modulate metabolic enzymes including LDHA, GLS1, and PC—implicated in both glycolysis (LDHA) and anaplerosis (GLS1 and PC). As a result, lactate production increases, while intracellular glutamine

declines accompanied by a robust increase of glutamate and aspartate, thus driving anaplerosis during accelerated glycolysis in endothelial dysfunction in PH. Adapted with permission from the *Journal of Clinical Investigation* [36]

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Fig. 3.

Potential therapeutic targets in PH based on mitochondrial and metabolic dysfunction of endothelial cells. This schematic of endothelial metabolic dysfunction in PH suggests several therapeutic targets (shown in *red circles* and listed on *top* of the figure) that have shown pre-clinical promise and in several cases are currently being tested in early-phase clinical trials. *αKG* α-ketoglutarate, *ER* endoplasmic reticulum, *GLS* glutaminase, *HIF* hypoxia-inducible factor, *MCD* malonyl-CoA decarboxylase, *MnSOD* manganese superoxide dismutase, *MnTBAP* Mn(III)tetrakis(4-benzoic acid)porphyrin chloride, *mROS* mitochondria-derived reactive oxygen species, *NFAT* nuclear factor of activated T cells, *PDH* pyruvate dehydrogenase, *PDK* pyruvate dehydrogenase kinase, *TK* tyrosine kinase. Adapted with permission from *Circulation Research* [67]