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Endothelial Cell Energy Metabolism, Proliferation, and Apoptosis in Pulmonary Hypertension

Weiling Xu* and Serpil C. Erzurum*

Departments of Pathobiology, Lerner Research Institute, Respiratory Institute, Cleveland Clinic, Cleveland, Ohio 44195, USA

Abstract

Pulmonary arterial hypertension (PAH) is a fatal disease characterized by impaired regulation of pulmonary hemodynamics and excessive growth and dysfunction of the endothelial cells that line the arteries in PAH lungs. Establishment of methods for culture of pulmonary artery endothelial cells from PAH lungs has provided the groundwork for mechanistic translational studies that confirm and extend findings from model systems and spontaneous pulmonary hypertension in animals. Endothelial cell hyperproliferation, survival, and alterations of biochemical-metabolic pathways are the unifying endothelial pathobiology of the disease. The hyperproliferative & apoptosis-resistant phenotype of PAH endothelial cells is dependent upon activation of signal transducer and activator of transcription (STAT) 3, a fundamental regulator of cell survival and angiogenesis. Animal models of PAH, patients with PAH and human PAH endothelial cells produce low nitric oxide (NO). In association with the low NO, endothelial cells have reduced mitochondrial numbers & cellular respiration, which is associated with more than a 3-fold increase in glycolysis for energy production. The shift to glycolysis is related to low levels of NO and likely to the pathologic expression of the pro-survival and pro-angiogenic signal transducer, hypoxia-inducible factor (HIF)-1, and the reduced mitochondrial antioxidant manganese superoxide dismutase (MnSOD). In this chapter, we review the phenotypic changes of the endothelium in PAH and the biochemical mechanisms accounting for the proliferative, glycolytic, and strongly pro-angiogenic phenotype of these dysfunctional cells, which consequently foster the panvascular progressive pulmonary remodeling in PAH.

I. INTRODUCTION

Pulmonary hypertension (PH) is identified clinically by pulmonary artery pressures (PAP) > 25 mm Hg at rest or > 30 mm Hg with exercise (107). PH is currently classified into five categories. Group 1 pulmonary arterial hypertension (PAH), Group 2 pulmonary hypertension with left heart disease, Group 3 pulmonary hypertension associated with lung diseases and/or hypoxemia, Group 4 pulmonary hypertension due to chronic thrombotic and/or embolic disease, and Group 5 pulmonary hypertension related to other miscellaneous conditions such as sarcoidosis, histiocytosis X or lymphangiomatosis (121). Group 1 PAH

*Corresponding authors Mailing Address: Weiling Xu, M.D. or Serpil C. Erzurum, M.D., Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave./ NC22, Cleveland, Ohio 44195, USA., Telephone: 216-445-7191, Fax: 216-636-0104, xuw@ccf.org or erzurus@ccf.org.

consists of three main subgroups, idiopathic pulmonary arterial hypertension (IPAH, group 1.1), familial pulmonary arterial hypertension (FPAH, 1.2), and pulmonary arterial hypertension related to associated conditions (APAH, 1.3) (121). Histopathologic abnormalities in PAH include neointima formation in pulmonary arteries and abnormal angiogenesis as evidenced by plexiform lesions with capillary-like channels near pulmonary arterioles. Plexiform lesions comprised of proliferative endothelial cells are characteristic in class I PAH. Most plexiform lesions in IPAH are seen at branching points of muscular arteries in the range of 200 μm (33, 59, 135). One can, however, see plexiform lesions immersed in the lung parenchyma, likely due to orientation of tissue planar sections such that one cannot see the connection of the plexiform lesion with the parent vessel. Some plexiform lesions may also arise from precapillary non-muscularized supernumerary or preacinar arteries, which taper off immediately after their take off rather than into arterial segments that progress with axial tapering towards capillaries. Jamison and Michel (59) described the presence of plexiform lesion in supernumerary artery branches in IPAH and in lungs of patients with congenital heart malformations; nevertheless, their reported lesional vessel diameter is also in the range of which Tudor and others have described. On the other hand, lectin-binding specificity in a plexiform lesion appears similar to the lectin-binding of small vessels in the rodent lung (123). However, lectin-binding may change in the pathologic endothelium in the plexiform lesion and the results must be validated in terms of lectin-specific binding in human “large” and “small” vessels.

In this chapter, we review the wealth of studies that provide strong evidence in favor of endothelial cell phenotypic changes in the pathogenesis of PH.

II. HYPERPROLIFERATIVE APOPTOSIS-RESISTANT ENDOTHELIAL CELLS IN PULMONARY HYPERTENSION

A. Hyperproliferative endothelial cells

Although PAH has been recognized for more than a century (157), proliferative endothelial cells were first postulated in the 1950s to participate in the vascular lesions of PAH based on pathologic features, particularly in plexiform and dilation lesions (53, 135). The endothelium lining the normal lung vasculature is characterized by significant heterogeneity (3, 68, 123). Voelkel and colleagues found that the plexiform lesions in lungs of patients with IPAH are composed of a homogeneous monoclonal cell population, while similar lesions present in lungs of patients with APAH due to Eisenmenger’s syndrome are uniformly polyclonal (74), the finding has not been replicated and thus it is not known if it relates to all IPAH patients. This provides evidence in favor of an endothelial proliferative process in PAH pathogenesis, and further suggests that PAH may arise from pathobiological processes that are common to neoplasia (134, 140). In support of neoplasia-like transformation, studies show that the tumor suppressor function of the transforming growth factor (TGF)- β family of signaling molecules is disordered as demonstrated by somatic microsatellite instability in the TGF- β receptor 2 and loss of receptor expression in pulmonary hypertension (153, 154). Consequently, the discovery that mutation of a TGF- β receptor family member, bone morphogenetic protein (BMP) receptor II (BMPRII), is one of the major genes underlying FPAH validated the idea of neoplastic-like transformation of the

vascular cells in PAH (38, 72). Heterozygous germline mutations in the BMPRII gene, localized to chromosome 2q33, have been identified in sporadic cases of IPAH (129). As members of the TGF- β superfamily of cytokines, BMPs transduce signals and induce growth arrest by binding to heteromeric complexes of type I and II receptors, e.g., BMPRII (85, 103). The commonly held hypothesis for familial PAH pathogenesis is that BMPRII mutations result in enhanced cell proliferation, leading to pulmonary artery remodeling, or alternatively that loss of BMP dependent survival signals may lead to endothelial cell apoptosis and selection of an apoptosis-resistant and pro-survival phenotype of endothelial cells. Given the low penetrance of the disease among individuals carrying the mutation, second-hits resulting from environmental exposure and/or genetic modifiers, required for pulmonary hypertension to develop still need to be investigated (4, 77, 137). Overall, the consensus emerging view is that PAH generally results from an imbalance between pulmonary vascular cell proliferation and susceptibility to apoptosis.

Relatively recent findings suggest that the clonal growth of endothelial cells in PAH occurs through an expansion of a stem-like/progenitor vascular cell population, which may arise from pulmonary arteries themselves or from the bone marrow (134). In the setting of IPAH, a single cell likely acquires the ability to expand and form a lesion, whereas in APAH, stimuli, such as shear stress, inflammation, or viral products, may mobilize and recruit vascular progenitor cells to form a polyclonal cell population as seen in the setting of congenital heart malformations, collagen vascular diseases, and HIV-infection. In support of progenitor cells in the proliferative process, there are greater numbers of CD34⁺ endothelial progenitor cells among IPAH endothelial cells as compared to control endothelial cells (12). The mechanisms involved in the endothelial cell proliferation in PAH might be similar to those present during blood vessel (vasculogenesis/angiogenesis) formation during embryo development. If endothelial cell proliferation in PAH occurs from stem cells located within the compromised pulmonary blood vessel wall, multiple progenitors may be activated or undergo transformation leading to the generation of multiple plexiform lesions simultaneously. However, it is yet unclear what may incite such a concerted activation (134).

Direct evidence in support of a proliferative endothelial cell population in plexiform lesions *in vivo* is provided by the detection of increased expression of proliferation markers (Ki-67/MIB-1)(32)(Fig. 1), angiogenesis and survival-related molecules (74, 131), e.g., vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR)-2, HIF-1 α and 1 β (131), and reduced expression of p27/kip1, a cell cycle inhibitory protein (32)(Tables 1 & 2). Cultures of primary endothelial cells derived from healthy and PAH lungs provide support for a generalized hyperproliferative nature of the PAH vascular endothelial cells. Pulmonary artery endothelial cells (PAEC) obtained from IPAH lungs have greater cell numbers in response to growth factors in culture due to increased proliferation as determined by bromodeoxyuridine (BrdU) incorporation and Ki-67 nuclear antigen expression (81)(Tables 1 & 2). IPAH endothelial cells have greater migration than control cells in *in vitro* experiments. Interestingly, although the PAEC from IPAH lungs grow more rapidly, they generate disorganized networks in *in vitro* Matrigel angiogenesis assays, suggesting a dysfunctional angiogenic potential (81)(Table 1). The perpetuation of more proliferative cells confirms the concept that IPAH arises from autonomous growth of phenotypically altered endothelial cells (133).

B. Resistance of endothelial cells to apoptosis

Endothelial cells in plexiform lesions display microsatellite site mutations and reduced protein expression of Bax, a pro-apoptotic member of the Bcl-2 gene family (153). Lack of expression of active caspase 3 (81) and diminished expression of PPAR γ , a member of a family of nuclear receptors/ligand-dependent transcription factors related to anti-proliferative and pro-apoptotic action, are identified abnormalities in endothelial cells in plexiform lesions *in vivo* (7)(Table 1). VEGF serves as a mitogenic and survival factor for endothelial cells and withdrawal of VEGF is associated with apoptosis of microvascular and normal pulmonary artery endothelial cells (42). All this suggests that the endothelial cells have a pro-survival phenotype as well as a greater proliferative nature *in vivo*. In a model capillary perfusion system with vascular endothelial cells lining the capillary tubes, VEGF receptor blockade leads to widespread death of most endothelial cells, but the population of cells that survive have a hyperproliferative phenotype and are apoptosis resistant (112). Thus, a substantial noxious injury to the vascular endothelium may favor repair via a select subset of proliferative and apoptosis-resistant endothelial cells. In fact, in a rodent model of angioproliferative pulmonary hypertension, blockade of apoptosis by caspase inhibition prevents the development of intravascular pulmonary endothelial cell growth and protects against development of severe pulmonary hypertension (127). In comparison to cells from control lungs, primary pulmonary artery endothelial cells obtained from IPAH lungs have decreased apoptosis as determined by caspase 3 activation and TdT-mediated dUTP nick end labeling (TUNEL) assay (81)(Table 1). IL-15, a pro-inflammatory cytokine, is a general inhibitor of apoptosis (24, 49), and promotes cell survival by preventing loss of Mcl-1, an anti-apoptotic member of the Bcl-2 family (102, 159). IL-15 gene expression is more than ~2-fold greater in IPAH pulmonary artery endothelial cells in culture as compared to normal cells, and IL-15 levels are higher in supernatants overlying IPAH cells than control endothelial cells (81). In parallel with the greater IL-15, IPAH pulmonary artery endothelial cells have higher Mcl-1 and Bcl-2 protein expression as compared to control cells in culture (81)(Table 1). Taken together, the cumulative studies substantiate that IPAH pulmonary artery endothelial cells have increased pro-survival factors and are resistant to apoptosis as compared to control endothelial cells.

C. STAT3 in the pro-survival and proliferative phenotype

Investigation of molecular mechanisms that are responsible for the pro-survival phenotype of PAH endothelial cells have led to the recognition of abnormalities in the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway. JAK-STAT signaling regulates many cellular processes including innate and adaptive immune function, development, cell proliferation, differentiation and apoptosis. Binding of a growth factor or cytokine ligand to a cell-surface cytokine receptor leads to the activation of kinases of the JAK family [JAK1, 2, 3, tyrosine kinases (Tyk)2] that then recruit and phosphorylate specific STATs. Once phosphorylated, STAT proteins form homo- or heterodimers (STAT1: STAT2, STAT1: STAT3), enter the nucleus, bind to specific DNA elements, and direct transcription (37, 87). Out of seven mammalian STAT proteins (STAT 1, 2, 3, 4, 5A, 5B, 6) identified so far, STAT3 has been recognized as essential for cell proliferation, survival and anti-apoptotic effects (1). Constitutively activated STAT3 has been identified in tumors and tumor-derived endothelial cells and is recognized as the primary signal transducer which

promotes invasive angiogenesis (43, 151). IL-6 (1), erythropoietin (Epo) (69), VEGF (16) or hepatocyte growth factor (HGF) (18) are the major ligands, which upon binding their cognate cell-surface receptors lead to STAT3 phosphorylation, homodimerization and/or heterodimerization (with STAT1). Upon activation and dimerization, STAT3 translocates to the nucleus, binds to DNA and activates transcription of genes that promote survival, proliferation, and angiogenesis (1, 43, 151), including HIF-1 α (62, 148), another robust signal transducer that promotes cell proliferation and augments angiogenesis (Fig. 2).

Proliferative and apoptosis-resistant PAH PAEC have been linked to persistent activation of STAT3. Studies of the monocrotaline (MCT)-induced pulmonary hypertension rat model reveal a reciprocal relationship between loss of caveolin-1 (Cav-1) in pulmonary endothelial cells and increased amounts of the activated tyrosine-phosphorylated STAT3 (pSTAT3) accompanied by positive nuclear immunostaining for proliferating cell nuclear antigen (PCNA), an indicator of DNA synthesis and cell proliferation (60, 83)(Table 2). The endothelial cells in the monocrotaline model have aberrant Golgi sorting of proteins and intracellular transport of signaling molecules (114), which have been implicated in STAT3 activation (93). In human PAH, immunohistochemistry shows an increase in activated (phosphorylated) STAT3 in endothelial cells within plexiform and concentric lesions *in vivo* (81)(Table 1). STAT3 persists in the phosphorylated and activated state in cultures of endothelial cells derived from PAH lungs, but not in cells from normal lungs (81). STAT3 inhibition blocks PAH endothelial cell proliferation in cultures (81), which supports the importance of STAT3 in the mechanisms leading to the proliferative endothelial phenotype (81, 119, 151). Inhibition of STAT3 in model systems prevents neointima formation by inhibiting proliferation and promoting apoptosis of neointimal smooth muscles cells (119). Mutations in BMPRII, which are linked to PAH, are associated with activation of STAT3 (38, 72, 103). BMPs induce growth arrest through intracellular signaling pathways of the Smad proteins (66), which act in part through inactivation of STAT3 (13, 155). Hence, loss of the inhibitory BMP pathway can be one mechanism that contributes to the increased activation of STAT3 in genetic causes of PAH (Fig. 2).

Although classically STAT3 is recognized as the transducer of signals to DNA to promote transcription of responsive genes, recent studies show that STAT3 is also present in the mitochondria where it regulates complexes I and II and subsequently functions to control cell respiration and metabolism (95, 146)(Fig. 2). Thus, while activated STAT3 in PAH endothelial cells certainly indicates changes in the cell proliferative and survival phenotype, it also implies a potential role in changes in cell energy metabolism, a phenomenon long recognized in neoplastic cells and recently identified in PAH as well.

III. ENERGY METABOLISM SHIFT IN PULMONARY HYPERTENSION

A. Glycolytic rate of endothelial cells *in vitro* and *in vivo*

Tumor cells exhibit reliance upon glycolysis and shift away from oxidative phosphorylation for cellular energy production, a phenomenon first described over 80 years ago and known as the Warburg effect (143). Characteristically, poorly differentiated cancers, cancers with higher incidence of metastases and/or rapidly growing tumors have higher glycolytic rates even under conditions where O₂ is plentiful (47, 147), which is the basis for measurement of

the glucose analog tracer [^{18}F] fluoro-deoxy-D-glucose (FDG) uptake by positron emission tomography (PET) to detect cancers (142, 143)(Fig. 3). The condition of aerobic glycolysis is not a unique feature of tumor cells but is also found in non-transformed rapidly proliferating cells when sufficient glucose is available (142). Thus, aerobic glycolysis has been linked primarily to rapid cell proliferation rather than to malignancy (142). Studies in human, avian, rodent and lamb pulmonary hypertension confirm that vascular cells in PH also switch to energy derived primarily from glycolytic metabolism (19, 25, 57, 88, 118, 126), and the metabolic changes are analogous to the alterations in cancer cell metabolism (45, 150)(Table 2).

In normal endothelial cells, adenosine triphosphate (ATP) is generated nearly equivalently by glycolysis and cellular respiration (36), accounting for a relative tolerance to hypoxia because of low oxygen demand and, compared to other cells, a relatively high glycolytic activity (36, 76). Cellular ATP content of PAH endothelial cells is similar to control cells under normoxia; however, under hypoxia, cellular ATP remains static in PAH endothelial cells but decreases significantly in control endothelial cells (150). The greater tolerance to hypoxia identifies that PAH endothelial cells have a lesser dependence upon cellular respiration for energy than control cells and suggests a predominant anaerobic cellular energy source. Glucose metabolism subserves the primary role for energy-requirements of PAH endothelial cells as shown by the more than ~3-fold greater glycolytic rate of PAH endothelial cells as compared to normal endothelial cells (Fig. 3). *In vivo* alterations in metabolism are verified by FDG PET scans, which reveal higher glucose metabolic activities in lungs of PAH patients than in healthy individuals (150)(Fig. 3)(Tables 1 & 2).

B. Mitochondria in pulmonary hypertension

Glycolysis, which yields 2 ATP per molecule of glucose versus 38 ATP with complete oxidation, can meet the enhanced ATP demand of proliferating cells but far less effectively (142). However, glycolysis is likely advantageous to rapidly growing cells as it renders them less dependent on oxygen, thereby improving their survival in an environment that may become hypoxic as cell numbers increase. Further, the transition to glycolysis by proliferating cells minimizes exposure to ROS, *i.e.* diminished oxidative metabolism (8, 100). Despite many years of investigation, however, it is still unclear as to what regulatory mechanisms transition proliferating cells from oxidative glucose metabolism to glycolysis (142). Recent studies indicate that glycolytic conversion of bioenergetics in cells is an early pre-transformation event and can be triggered by p53, a gene commonly mutated in cancers (45, 84, 109). It has also been speculated that decreased mitochondrial function may be a primary stimulus to glycolysis (76, 86). There is evidence to favor primary abnormalities in mitochondrial function in the metabolic shift to glycolysis in PH, as well as evidence in some systems that suggest that mitochondrial changes are secondary to alterations of endothelial function.

Mitochondria are essential to cellular energy production in all higher organisms adapted to an oxygen-containing environment. Cellular respiration consumes oxygen and glucose to make energy-storing molecules of ATP. This occurs primarily through oxidative phosphorylation carried out by the electron transport chain which is composed of five multi-

subunit complexes located in the mitochondrial inner membrane. The electrochemical gradient used by mitochondrial F₀F₁ ATP synthase to synthesize ATP from adenosine diphosphate (ADP) is generated by the proton pump action performed by Complexes I, III, and IV of the respiratory chain. The proton pumping is accompanied by electron shuttling, whereby Complexes I and II, along with the flavoprotein – ubiquinone oxidoreductase, transfer electrons from different sources to ubiquinone (coenzyme Q). The electrons are then transferred sequentially to Complex III, cytochrome *c*, Complex IV, and finally to molecular oxygen, the terminal electron acceptor (Fig. 4). Thus, mitochondria are the primary oxygen demand in the body, accounting for roughly 90% of cellular oxygen consumption. Under limiting oxygen conditions, cells generally turn to glycolysis to generate energy.

Mitochondrial defects have been long recognized in the pathophysiology of a wide variety of human diseases, e.g., Huntington disease, Parkinson disease, Friedreich's ataxia, as well as in cancer and recently in PH (113). Studies of pulmonary hypertension in avian, rodent species and lambs identify intrinsic deficiencies in mitochondrial function (19, 118, 126) (Table 2). Site-specific defects in the electron transport chain within complex I and III have been identified in avian spontaneous idiopathic pulmonary hypertension; the lower respiratory chain coupling, inefficient use of oxygen and greater generation of reactive oxygen species (ROS) is directly related to the development of pulmonary hypertension in avian species (25, 57, 126)(Tables 2 & 3). Similarly, Fawn Hooded rats, a spontaneously pulmonary hypertensive rodent strain, have mitochondrial abnormalities including dysmorphic mitochondria with reduced expression of electron transport chain components (complex I, III and IX), reduced expression of MnSOD, depressed mitochondrial ROS production (H₂O₂) and activation of pyruvate dehydrogenase kinase (PDK), which shift metabolism away from oxidative phosphorylation toward glycolysis. The abnormalities in mitochondrial function are associated with normoxic activation of HIF-1 α . Strikingly, dichloroacetate (DCA), a mitochondrial PDK inhibitor, reverses the pulmonary hypertension of Fawn Hooded rats, providing a mechanistic relationship between mitochondrial function and the pathogenesis of pulmonary hypertension (19, 88)(Fig. 4)(Table 2). The decreased expression and activity of mitochondrial enzymes required for carnitine metabolism in lambs also leads to mitochondrial dysfunction, including decreased MnSOD expression and increased uncoupling protein-2 expression, which leads to increased glycolysis (decreased pyruvate, increased lactate, and a reduced pyruvate/lactate ratio), endothelial dysfunction and ultimately pulmonary hypertension (118)(Table 2).

Decreased mitochondrial function is also present in endothelial cells obtained from human PAH lungs (19, 150). The reduction of MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide], a simple assay of cells which requires functional mitochondrial dehydrogenase activity, is strikingly lower in PAH endothelial cells than control, which indicates either fewer mitochondria or decreased mitochondrial function (150). Quantitation of mitochondria in electron microscopy images of cells and southern analysis of mitochondrial DNA (mtDNA) from endothelial cells in culture reveal decreased mitochondrial numbers in PAH cells as compared to control, but no discernible difference in mitochondrial morphology (Fig. 5). Careful measure of PAH endothelial cells *in vitro* show that they have lower than normal oxygen consumption for state 3 and state 4 respiration with glutamate-malate or succinate as substrate (Table 3). 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 in PAH PAEC than in control, while Complex III activity is similar among control and PAH endothelial cells. However, the coupling between oxygen consumption and ATP production, the respiratory control index (RCI) of mitochondrial function, is similar among PAH and controls. Thus, human PAH endothelial cells appear to suffer primarily from lower mitochondrial numbers, which results result in greater reliance upon glycolysis, hence less ATP generated by cellular respiration.

C. ROS and antioxidants in pulmonary hypertension

Abnormalities in mitochondria regulation of ROS are also present in animal and human PH. Mitochondria are the largest producer of ROS, which also occurs through the process of cellular respiration in which one-electron reduction of oxygen to superoxide radical is followed by formation of hydrogen peroxide, as opposed to the four-electron reduction of oxygen to water (139). ROS including hydroxyl radicals ($\bullet\text{OH}$), superoxide ($\text{O}_2\bullet^-$) and hydrogen peroxide (H_2O_2) (30, 128) are thus by-products of normal cellular metabolism. The mitochondria avoid the adverse effects of ROS by minimizing oxidant damage to biological molecules through an integrated and abundant antioxidant system of enzymatic and expendable soluble antioxidants including MnSOD (120, 144) (Fig. 4). Many studies indicate that ROS levels are increased in human PH, whether idiopathic or associated with other lung diseases, and that ROS contribute to the vascular remodeling (21, 35, 64, 71, 145) (Table 2). In addition, most studies indicate a loss of mitochondrial MnSOD activity in human and model systems of PH, an event that not only increases ROS but also contributes, via consumptive reactions, to the loss of nitric oxide (NO) that is common to PH (Fig. 4). The levels of superoxide dismutase (SOD) in PH lung tissues is directly related to exhaled NO and inversely related to pulmonary artery pressures (80)(Table 2). Strong data in support of ROS and loss of MnSOD in the pathogenesis of PAH is derived from studies in which antioxidants prevent or abrogate PAH (17, 21, 35, 64, 71, 145). For example, the antioxidant dimethylthiourea inhibits right heart dysfunction usually induced by a 10-day exposure to hypoxia in rats (54, 70, 73) and significantly abrogates the pulmonary hypertensive response (54). PH syndrome in avian species, with reduced respiratory chain coupling, inefficient use of oxygen and higher generation of ROS (25, 57, 126), is successfully treated by implanting nonenzymatic antioxidant pellets of vitamin E after chickens hatch (20). The incidence of avian PH syndrome is decreased, and antioxidant capacity increased, in vitamin E-treated chickens (20). Similarly, N-acetyl-L-cysteine, which serves as a precursor to the non-enzymatic antioxidant glutathione, reduces pulmonary hypertension, right ventricular hypertrophy, and pulmonary vascular media thickening in a hypoxia-induced rodent model of PH (55). In an interventional study in a neonatal PAH lamb model, recombinant human SOD administered intratracheally reduced both vasoconstriction and oxidation (71).

Although it seems paradoxical, mitochondrial numbers and specific functions may be reduced while mitochondrial ROS production is increased. For example, growing evidence from diabetes indicates that oxidative stress is increased due to overproduction of reactive oxygen species (ROS) by mitochondria, decreased efficiency of antioxidant defenses, and related mitochondrial DNA mutations and abnormalities in function (2, 6, 141). The

generation of ROS may be increased due to uncoupling of mitochondrial electron transport to ATP synthesis (111). Although ROS levels are increased in human PH, ROS levels among animal models are inconsistent across studies. Further work is required to understand the contribution of ROS and the types of ROS, e.g., superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), to the vascular remodeling (Table 2).

Altogether, the cumulative data point to decreased mitochondrial function, whether related to lower numbers of mitochondria and/or an intrinsic impairment of function, as a pathophysiologic hallmark of pulmonary hypertension across species that is associated with the emergence of a glycolytic hyperproliferative apoptosis-resistant endothelial phenotype. Intriguingly, a recent case report of the development of PAH in a child with the primary mitochondrial genetic disorder of MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke) that is due to a point mutation of the mitochondrial tRNA^(Leu) gene (m. 3243A>G) provides one proof of concept that mitochondrial dysfunction can cause the syndrome of PAH in humans (122).

D. Nitric oxide and metabolism in pulmonary endothelial cells

Abnormalities in NO have been established in the pathogenesis of pulmonary hypertension (10, 15, 31, 39, 61). Vasodilatory effects of NO are mediated by signal transduction primarily through activation of soluble guanylate cyclase to produce guanosine 3',5'-cyclic monophosphate (cGMP) (124). There is conclusive evidence from animal models of pulmonary hypertension, mice genetically deficient in the endothelial NO synthase and/or in the pathways required for generation of cofactors or regulatory effectors of NO synthesis, and complementation studies with gene transfer of NOSs for the concept that NO is a critical determinant in the development of pulmonary hypertension (26, 56, 91). Therapies that augment NO reduce pulmonary vascular resistance in PAH (10, 15, 31, 61, 130), and inhibit development of hypoxic PH (31). Much more than a regulator of pulmonary vascular tone, NO regulates cellular bioenergetics through effects on glycolysis, oxygen consumption by mitochondria, and mitochondrial biogenesis (29, 90, 98, 99). Hence, a detailed review of NO is required to understand the phenotypic changes in PH endothelial cells.

NO is synthesized endogenously by NO synthases (NOS) in mammalian cells (EC 1.14.13.39). Three isoforms of NOS, which are products of separate genes (97, 125), have been identified, including neuronal (nNOS or NOS I), endothelial (eNOS or NOS III) and iNOS or NOS II (46, 97, 125)(Table 4). These enzymes convert L-arginine to NO and L-citrulline in a reaction that requires oxygen, NADPH, and cofactors FAD, FMN, tetrahydrobiopterin (BH_4), calmodulin and iron protoporphyrin IX. The mice genetically deficient in eNOS develop PH and right heart failure (40), but alterations in levels of cofactors also cause PH (Table 2). The cofactor BH_4 is essential for NOS enzymatic activity (65)(Fig. 6). Under conditions of limiting BH_4 , NO generation stops and superoxide production by NOS enzymes predominates as a result of the loss of enzymatic coupling between the reduction in molecular oxygen and oxidation of L-arginine (5, 34). The hyperphenylalaninemic mutant mouse, which has deficient BH_4 production due to reduced activity of the rate limiting enzyme in BH_4 synthesis GTP-cyclohydrolase I (Fig. 6), provides proof of the concept that deficiency of NOS cofactor leads to reduction of NO

synthesis and development of pulmonary hypertension. Definitive evidence for the NO pathway in the development of PH in this mouse is provided by crossing the hyperphenylalaninemic mutant mice to mice with endothelial-targeted over-expression of GTP-cyclohydrolase I, which restores NO synthesis and rescues the progeny from developing pulmonary hypertension (34, 67, 96)(Table 2).

Compartmentalization of eNOS in caveolae and other subcellular sites positions the enzyme for interaction with regulatory proteins and phosphorylation-dependent events that also determine eNOS activity and functions (Figs. 5 & 7). In this context, Cav-1^{-/-} mice, which lack caveolae, have high NO levels due to loss of eNOS inhibition by cav-1; but lack of the proper spatial orientation of NO production also results in hyperproliferative endothelial cells and pulmonary hypertension/heart failure in the genetically deficient mice (158)(Table 2). Spatial orientation of eNOS in the outer mitochondrial membrane is regulated through a stretch of five basic amino acid residues (RRKRR) within the autoinhibitory domain of eNOS; this contributes to anchoring the enzyme to the outer mitochondrial membrane (Fig. 7). Intriguingly, deletion of the pentabasic amino acid sequence in eNOS does not impair NO production, but results in dramatic depletion of eNOS from mitochondria and increases the rate of oxygen consumption by mitochondria in the cell (44). Thus, although not completely defined, the L-arginine/NO pathway is a major regulator of metabolism in the cell.

NO binds to several targets and inhibits their functions within the mitochondrial respiratory chain, e.g. Complex I, Complex II and Complex IV (Fig. 4) (9, 108). On this basis, mitochondrial function and cellular respiration might be expected to increase in the condition of low NO as in PAH. However, NO has other long-term dominant effects on mitochondrial biogenesis and function in cells (29, 90, 98, 99). Long-term exposure of cells in culture to NO donors induces mitochondrial biogenesis and enhances coupled respiration, oxygen consumption and ATP generation mediated by NO/cGMP-dependent processes, through the induction of peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α), nuclear respiratory factor 1 (NRF-1), and mitochondrial transcription factor A (Tfam)(98, 99). Thus the role of NO on mitochondria likely is dependent on amounts of NO, the site of NO effect and the length of exposure to NO (Table 5). The first evidence of the role of eNOS in mitochondrial biogenesis was provided by the eNOS-deficient mice, which develop profound PH under hypoxia (40), have reduced mitochondria content in a wide range of tissues and significantly lower oxygen consumption and ATP content (29, 90, 98, 99).

Deficiency of NO is well described in patients with PAH (10, 15, 61, 64, 78, 101). Pulmonary and total body NO are lower in PAH patients as compared to healthy controls (50, 64, 78, 101, 149). Several mechanisms for the lower NO production by endothelial cells in PAH have been advanced. Immunohistochemical analysis has suggested that PAH is associated with diminished expression of the eNOS isoform (48, 138). However, others studies have shown only a modest decrease in small arterioles, with strong immunostaining in almost all plexiform lesions in pulmonary hypertension compared with normal lung tissues (79, 138, 149)(Fig. 8). NO production by eNOS is lower than normal in the hyperproliferative, apoptosis-resistant PAH endothelial cells *in vitro* (149, 150)(Tables 1 &

2). Western analyses identify normal levels of eNOS expression in PAH endothelial cells (149). On the other hand, the pulmonary artery endothelial cells derived from PAH lung have higher arginase II expression, an enzyme which regulates NO biosynthesis through effects on arginine (Fig. 6)(Table 1). High levels of arginase II expression is localized by immunostaining to pulmonary endothelial cells in PAH lungs.

Irrespective of the causes for the low level of NO production in PAH, the low NO levels contribute to the decreased mitochondrial function in PAH endothelial cells (150). The primary abnormality identified in human endothelial cells from PAH lungs is a reduction in total numbers of mitochondria per cell. Mitochondrial regeneration occurs in the cells with exposure to NO donors for several days. Mitochondrial function as determined by dehydrogenase activity measured by MTT substantially increases following NO donors; likewise Complex III-1 and cytochrome *c* protein levels increase ~2-fold with NO donors. Thus, a wealth of data support the mechanistic link between abnormalities of NO production and the metabolic abnormalities of endothelial cells across species, models of PH and in spontaneous PH (150).

E. Hypoxia-inducible factors in the metabolic shift to glycolysis

Hypoxia-inducible factor (HIF)-1, a heterodimeric transcription factor which has an oxygen-sensitive alpha subunit (HIF-1 α) and constitutive HIF-1 β subunit, enables cells to respond/adapt to environmental oxygen availability (115). HIFs regulate genes that control energy metabolism, erythropoiesis, vasomotor tone, and angiogenesis (116). Furthermore, HIF-1 α plays a pathologic role in tumor angiogenesis and neoplastic invasion (116) and is directly involved in the metabolic shift of cancer cells towards glycolysis, which underlies the Warburg phenomena (132, 143)(Fig. 9). In this context, it is not surprising that HIF-1 α and 1 β expression, and the downstream HIF-activatable gene VEGF, have been identified within endothelial plexiform lesions by immunohistochemistry. The immunolocalization to vascular lesions suggests that HIF-dependent signaling contributes to the hyperproliferative and metabolic abnormalities in PAH (131).

The potential pathogenic role of HIF-1 α in pulmonary hypertension is validated by genetic deletion of HIF-1 α (156) or HIF-2 α (23) heterozygous mice, which have less hypoxic pulmonary hypertension when compared with littermates. Increased mitochondrial ROS production by inhibition of cellular respiration through mutation of complex II increases HIF-1 α stabilization and activates numerous intracellular signaling and transcriptional pathways (27, 28, 51, 117, 152). Thus, mitochondrial abnormalities can direct HIF activation. Abnormalities in HIF-1 α and mitochondria pathology underlie the pulmonary hypertension observed in the Fawn-Hooded rat model of spontaneous disease (19). In the Fawn-Hooded rat, decreased MnSOD activity is causally associated with HIF-1 α activation and the consequent PH (19). In model systems, knockdown of MnSOD is sufficient for HIF-activation (63). Alternatively, abnormalities of NO that are present in PAH endothelial cells may contribute to the activation of HIF in PAH. NO has opposing effects on HIF activation depending on oxygen availability (82). Under hypoxia, HIF-1 α is destabilized by low levels of NO (75, 82). The inhibitory effect of low concentrations of NO in cells under hypoxia is attributed to inhibition of mitochondrial respiration (82). Under hypoxia, blockade of

cellular respiration by NO enables higher overall intracellular O₂ availability for increased degradation of HIF-1 α (52, 92, 94, 104, 106)(Fig. 9). It is tempting to speculate that loss of NO production in PAH endothelial cells may enable an unchecked HIF-activation in PAH endothelial cells. However, decreased NO synthesis, loss of MnSOD activity as well as HIF-activation are likely all involved in the development of the glycolytic and proliferative phenotype of PAH endothelial cells.

IV. INVESTIGATIONS IN THE FUTURE

Despite the accumulating wealth of information, further work is needed to address several fundamental questions regarding the pathogenesis of pulmonary hypertension. Some areas of study include:

- In genetic PAH, are there second-hits that lead to development of pulmonary hypertension? If so, identification of the secondary genetic targets is needed.
- More studies are needed to reconcile the possible mechanisms by which multiple monoclonal plexiform lesions may develop.
- The specific reactive species that are involved in human PAH remodeling are still not clear. The relationship between HIF-1 α activation and ROS and/or loss of MnSOD is an area that requires further focus of study given potential for targeted therapies.
- Clarification of the type(s) and causes of mitochondrial dysfunction in PH and how metabolic changes may participate in genesis of the pulmonary vascular disease.

V. CONCLUSION

Accumulating evidence from several groups have identified alterations of NO production, ROS, MnSOD activity, and cell bioenergetics as universal hallmarks of PH across species including murine, rodent, avian and human. These abnormalities are closely linked to pathologic signal transduction abnormalities including STAT3-activation and HIF-expression. The characteristic hyperproliferative and apoptosis-resistant endothelial cells are found in all models of PH and in human PAH. The accompanying metabolic shift to glycolysis in the proliferative endothelial cells affirms that neoplasia-like processes are operative in the genesis of the endothelial cell phenotype. Ultimately, understanding the biochemical-metabolic, molecular and physiologic underpinnings of PH will enable the design of targeted therapies that aim at the heart of these abnormalities in order to retard or reverse pulmonary vascular remodeling events.

ABBREVIATIONS

ADP	adenosine diphosphate
APAH	pulmonary arterial hypertension related to risk factors or associated conditions

ATP	adenosine triphosphate
BMP	bone morphogenetic protein
BMPRII	bone morphogenetic protein receptor type II
BrdU	bromodeoxyuridine
Cav-1	caveolin-1
cGMP	guanosine 3',5'-cyclic monophosphate
CrAT	carnitine acetyltransferase
DCA	dichloroacetate
eNOS	endothelial nitric oxide synthase
Epo	erythropoietin
FPAH	familial pulmonary arterial hypertension
FDG	[¹⁸ F] fluoro-deoxy-D-glucose
GSH	reduced glutathione
GSHPx	glutathione peroxidase
H₂O₂	hydrogen peroxide
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
IPAH	idiopathic pulmonary arterial hypertension
JAK	Janus kinase
MCT	monocrotaline
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke
MnSOD	manganese superoxide dismutase
mtDNA	mitochondrial DNA
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NO	nitric oxide
NOS	nitric oxide synthases
O₂•⁻	superoxide
ONOO⁻	peroxynitrite
PAEC	pulmonary artery endothelial cells

PAH	pulmonary arterial hypertension
PAP	pulmonary artery pressure
PCNA	proliferating cell nuclear antigen
PDK	pyruvate dehydrogenase kinase
PET	positron emission tomography
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1 α
PH	pulmonary hypertension
PHS	pulmonary hypertension syndrome
pSTAT3	phosphorylated STAT3
RCI	respiratory control index
REDOX	reduction-oxidation reactions
ROS	reactive oxygen species
STAT	signal transducer and activator of transcription
SOD	superoxide dismutase
TUNEL	TdT-mediated dUTP nick end labeling
Tyk	tyrosine kinases
VEGF	vascular endothelial growth factor

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Figure 1. Ki-67 in IPAH lung.

IPAH plexiform lesion shows intense immunoreactivity of proliferative endothelial cells for nuclear Ki-67 antigen (arrows) in low power view (B) and high power view (C). H&E staining of the plexiform lesion (A). (Original magnification: A and B: $\times 200$; C: $\times 400$)

Figure 2. Signal Transduction pathway for STAT3.

IL-6, Epo, or growth factors bind their receptors on endothelial cells and induce a tyrosine (Tyr) phosphorylation cascade, which involves activation of receptor-associated Janus kinase family tyrosine kinases (Jak/Tyk) by cross-phosphorylation. STAT3 phosphorylation, homodimerization and/or heterodimerization (with STAT1) and translocation to the nucleus, is followed by binding to DNA elements to activate transcription of genes that promote angiogenesis and cell survival, such as vascular endothelial growth factor (VEGF) and HIF-1 α . Serine (Ser) phosphorylation of STAT 3 appears to be required for its action in mitochondria. Bone morphogenetic proteins (BMPs) bind to bone morphogenetic protein receptor type II (BMPRII), and induce growth arrest through intracellular signaling pathways of the Smad proteins, which act in part through interaction with, and inactivation of, STAT3. GAS, γ -activated site; SIE, cis-inducible element.

Figure 3. PET/CT image, glycolytic rate in vitro, and glucose metabolic activities in vivo. A – B: CT image of lung of IPAH (A) and healthy control subject (B). C – D: PET image of lung of IPAH (C) and healthy control (D). E: Glycolytic rate from IPAH PAEC ($n = 5$) was higher than healthy controls ($n = 3$) (* $P < 0.01$). F: Glucose metabolic activities in lungs of IPAH patients were higher than healthy controls (* $P < 0.01$) in 4 IPAH patients (subjects 4 – 7) as compared to 3 healthy controls (subjects 1 – 3). To account for variations in lung tissue density and [¹⁸F] fluoro-deoxy-D-glucose (FDG) distribution between subjects, standardized uptake value (SUV) of each region of the lung was normalized for lung tissue fraction [LTF, determined from lung CT Hounsfield units (HU)] and HU of liver using the formula: $SUV_{normalized} = SUV \text{ of each region of the lung} / LTF \text{ lung of same region} / \text{mean HU of liver (150)}$.

Figure 4. Mitochondria oxidative phosphorylation related to NO production, MnSOD activity and glycolysis.

Complexes I and II receive electrons (depicted with an e⁻) from different sources and transfer them to ubiquinone (coenzyme Q, CoQ). The electrons are then transferred sequentially to Complex III, cytochrome *c* (Cyt C), Complex IV, and finally to molecular oxygen, the terminal electron acceptor. All multisubunit complexes of the respiratory chain are located in the mitochondrial inner membrane. The proton (depicted by H⁺) pumping is performed by complexes I, III, and IV, and generates an electrochemical gradient which is used by complex V to synthesize adenosine triphosphate (ATP). NO binds to several targets within the mitochondrial respiratory chain, e.g. Complex I, Complex II and Complex IV, and inhibits their function. Superoxide (O₂^{•-}), generated from mitochondrial complexes I and III, is converted to hydrogen peroxide (H₂O₂) by manganese superoxide dismutase (MnSOD). Glucose is converted to pyruvate and lactate via glycolysis. Under aerobic conditions, pyruvate in most cells is further metabolized via the tricarboxylic acid (TCA) cycle. Under anaerobic conditions and in erythrocytes under aerobic conditions, pyruvate is converted to lactate which is transported out of the cell into the circulation. Dichloroacetate (DCA) inhibits pyruvate dehydrogenase kinase and thereby activates pyruvate dehydrogenase with an attendant increase in intra-mitochondrial acetyl coenzyme A (acetyl

CoA). This drives TCA cycle and increases availability of electron donors for complex I and II within the mitochondria. ADP, adenosine diphosphate.

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Figure 5. Ultrastructure of PAEC from IPAH patient and healthy control. A: Ultrastructure of PAEC in 3-dimensional Matrigel *in vitro*.

Healthy control PAEC with junctions & spaces between plasma membranes of 2 adjacent cells. Mitochondria (M) are close to caveolae (arrowheads) in control PAEC (scale bar: 250 nm). B – C:

Electron microscopy images reveal decreased mitochondrial numbers in IPAH endothelial cells compared to control. Ultrastructure detail of pulmonary artery endothelial cells from IPAH (C) and healthy control (B)(scale bar: 1 μ m). ER, endoplasmic reticulum; M, mitochondria; N, nucleus.

Figure 6. Pathways in NO Metabolism.

NOS converts L-arginine to NO and citrulline. ADMA, asymmetric dimethylarginine; BH₄, tetrahydrobiopterin; cGMP, guanosine 3',5'-cyclic monophosphate; GSH, reduced glutathione; GSNO, S-nitrosoglutathione; L-Arg, L-arginine; L-Cit, L-citrulline; MMA, monomethyl-L-arginine; NADPH, nicotinamide adenine dinucleotide phosphate; NO[•], nitric oxide; NOS, nitric oxide synthases; NO₂⁻, nitrite; NO₃⁻, nitrate; O₂⁻, superoxide; OONO⁻, peroxynitrite; SOD, superoxide dismutase.

Figure 7. eNOS interactions with proteins in membrane-bound organelles affects activation and function.

Cav-1 is the principal component of caveolae but also found in the outer mitochondrial membrane. Cav-1 and eNOS co-localize at caveolae. Hsp90 interacts with and activates eNOS. eNOS is anchored to mitochondria by a pentabasic amino acid sequence (RRKRRK). Agonist-receptor binding triggers kinase cascades to phosphorylate eNOS as shown in Figure.

Figure 8. eNOS and pSTAT3 staining in plexiform lesion from lung of IPAH patient. IPAH vascular plexiform lesion have strong positive eNOS immunoreactivity in endothelial cells (A, arrows). Strong positive immunoreactivity of pSTAT3 is also present in endothelium in plexiform lesions (C, arrows). Immunohistochemical staining for CD31 in the laminar lining cells of the vessels confirms endothelial phenotype of cells (B & D). (Original magnification: $\times 200$)

Figure 9. HIF regulation by hypoxia, STAT3, NO and ROS.

Under normoxia, prolyl hydroxylases (PHD)-containing proteins hydroxylate hypoxia-inducible factor (HIF)-1 α , which mediates von Hippel-Lindau tumor suppressor (pVHL) binding, and HIF ubiquitination and proteasomal degradation. Under hypoxia, HIF-1 α stabilizes, dimerizes with HIF-1 β and interacts with hypoxia-responsive elements (HRE) leading to expression of genes, which are active in conversion to anaerobic metabolism and increasing oxygen delivery to tissues. Under normoxic conditions, HIF-1 α is stabilized by reactive oxygen species (ROS), knockdown of MnSOD, or high levels of NO, while hypoxia, MnSOD overexpression or low levels of NO destabilize HIF-1 α . Activation of signal transducer and activator of transcription (STAT)3 increases HIF-1 α mRNA expression and protein stability. ET-1, endothelin-1; eNOS, endothelial NOS; Epo; erythropoietin; SDF, stromal cell-derived factor.

Table 1.

Endothelial Cell Abnormalities in human PAH lungs and/or in primary pulmonary artery endothelial cells derived from human PAH lungs

Abnormalities	Cells <i>in vitro</i>	Lungs <i>in vivo</i>
Proliferation	Ki-67 (81), BrdU (81)	Ki-67 (32)
Apoptosis-resistant	Caspase 3 (81), Bcl-2 (81), Mcl-1 (81), TUNEL (81)	Bax (153)
REDOX		MnSOD (21, 80), Nitrotyrosine (21)
Signal transduction	pSTAT3 (81)	pSTAT3 (81), HIF (131)
Metabolism	Mitochondria function/Warburg (150)	FDG-PET/Warburg (150)
Angiogenesis	Tube formation in angiogenesis assay (12, 81),	VEGF, VEGFR2 (131), Monoclonal proliferation (74)
Nitric Oxide	eNOS (149), Arginase II (149), NO production (64, 149, 150)	eNOS (149), Arginase II (149), Exhaled NO (64, 78, 101)

Table 2.

Cellular and metabolic alterations in pulmonary hypertension

Pulmonary hypertension	Metabolism	Nitric oxide	Antioxidants & ROS	Proliferation
Human (21, 80, 150)	Cellular respiration↓ Glycolysis↑	NO↓	MnSOD↓ SOD activity↓ 8-hydroxy guanosine↑	↑
Broilers (58, 126)	Cellular respiration↓		GSH↓ H ₂ O ₂ generation↑	
Cav-1 ^{-/-} mice (14, 110, 158)	Lipid uptake↑	NO↑		↑
CrAT ^{-/-} lambs (118)	Cellular respiration↓ Glycolysis↑	NO↓	MnSOD↓ O ₂ • ⁻ ↑	
eNOS ^{-/-} mice (90, 98)	Cellular respiration↓	NO↓		↑
Fawn Hooded rats (11, 19, 136)	Cellular respiration↓ Glycolysis↑	NO↓	MnSOD↓ H ₂ O ₂ ↓ O ₂ • ⁻ ↓	↑
GTP-cyclohydrolase I deficient mouse (96)		NO↓		↑
MCT rat (41, 60, 89, 105)	Glycolysis↑	NO response↓	SOD↓ GSHPx↓ catalase↓	↑

Table 3.

Oxygen consumption of pulmonary artery endothelial cells from IPAH patients (150) and heart muscle from chickens with pulmonary hypertension syndrome (PHS) (126)

	Pulmonary artery endothelial cells (<i>natom of O /min/10³ cells</i>)			Heart muscle (<i>natom of O/min/mg protein</i>)		
	Control	IPAH	<i>P</i> value	Control	PHS	<i>P</i> value
Glu-Mal or Pyr-Mal						
state 4	2.0 ± 0.1	1.2 ± 0.1	<i>P</i> < 0.01	45.6 ± 3.8	57.2 ± 7.9	
state 3	5.4 ± 0.4	3.3 ± 0.3	<i>P</i> < 0.01	162.1 ± 13.2	118.5 ± 6.6	<i>P</i> = 0.01
RCI	2.74 ± 0.20	2.65 ± 0.16		3.60 ± 0.25	2.18 ± 0.16	<i>P</i> < 0.01
Succinate						
state 4	3.5 ± 0.2	1.8 ± 0.1	<i>P</i> < 0.01	85.1 ± 10.6	92.6 ± 18.4	
state 3	9.4 ± 0.1	4.7 ± 0.6	<i>P</i> < 0.01	216.7 ± 30.3	144.9 ± 17.3	<i>P</i> = 0.05
RCI	2.71 ± 0.23	2.60 ± 0.38		2.52 ± 0.09	1.69 ± 0.18	<i>P</i> < 0.01

Glu-Mal, Glutamate-malate; Pyr-Mal, pyruvate-malate. Respiratory control index (RCI) was calculated by dividing oxygen consumption rate at State 3 by State 4.

Table 4.

Nitric oxide synthases

Isoform	Chromosomal localization (Human)	Expression	Activity	Localization (Lung)
nNOS (I)	12q24.2	Constitutive	Ca ²⁺ dependent	Nonadrenergic, noncholinergic inhibitory neurons
iNOS (II)	17cen-q12	Inducible	Ca ²⁺ independent	Airway epithelial cells
eNOS (III)	7q35–36	Constitutive	Ca ²⁺ dependent	Pulmonary endothelial cells

Table 5.

NO effects on mitochondria and cellular respiration

Molecular mechanisms	Targets	Effects
*Tyrosine nitration (NO ₂ -Tyr)(22)	Complex I	Inactivation /decreased respiration
S-nitrosylation of thiol (-SNO) (22, 108)	Complex I	Inactivation /decreased respiration
Iron-nitrosyl [Fe (II)-NO]	Complex IV (108)	Inactivation /decreased respiration
	Guanylate cyclase (98)	Activation/enhanced mitochondrial biogenesis and respiration
*Iron-sulfur clusters (108)	Complex II	Inactivation /decreased respiration

* Most frequent under condition of high NO.