Nitric Oxide in Pathophysiology and Treatment of Pulmonary Hypertension

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1. Introduction

All conditions causing pulmonary hypertension (PH) are characterized by three major changes in the pulmonary vasculature: vasoconstriction, vascular remodeling, and thrombosis [1,2,3]. Vascular remodeling includes muscularization of normally non-muscular peripheral pulmonary arteries, increase in medial wall thickness of muscular arteries, and increase in vascular connective tissue such as collagen and elastin [1,2,3]. Imbalance of vasoconstrictive and vasodilatory mediators might explain the increased vascular tone [1,2,3]. Endothelial cells synthesize and release prostacylin and nitric oxide for vasodilation as well as endothelin and thromboxane for vasoconstriction. Approved treatments for pulmonary arterial hypertension (PAH) include prostacyclins, endothelin receptor blockers, and phosphodiesterase-5 inhibitors as well as inhaled NO for persistent pulmonary hypertension of the neonate (PPHN) [2].

Studies have demonstrated that short- and long-term NO inhalation improves arterial oxygenation and reduces pulmonary artery (PA) pressure in animal models of PH [4,5,6,7,8,9,10] and clinical disease such as post-operative congenital heart disease [11,12], chronic obstructive pulmonary disease (COPD) [13], pulmonary fibrosis [14], and acute respiratory distress syndrome (ARDS) [15]. In chronic hypoxia-induced PH in rats, we showed that low-dose NO (less than 5ppm) induces a submaximal reduction in pulmonary artery pressure, which does not correlate with the severity of pulmonary vascular changes [4]. Clinically, the effect of inhaled NO is based on pulmonary vasorelaxation. In experimental settings, NO inhibits vascular smooth muscle cell proliferation directly through regulating protein kinases modulating gene expression for cell growth and/or indirectly through reducing pressure on the vascular cells by cyclic guanosine-3',5'-monophosphate (cGMP) dependent

vascular relaxation. In this chapter we will discuss NO and its regulation and function with special references to the development of PH as well as pulmonary vascular reactivity in PH.

2. Biological effects of NO

2.1. NO acts through the sGC pathway and S-nitrosylation of target proteins

NO activates soluble guanylyl cyclase (sGC) stimulating cGMP production and subsequent activation of cGMP-dependent protein kinase (PKG). This sGC-cGMP-PKG pathway plays a major role in NO-mediated regulation. In addition to this pathway, NO directly binds to proteins and induces conformational changes with subsequent functional alterations, like phosphorylation. Thus, S-nitration is also called S-nitrosylation, the term which emphasizes a biological effect of the chemical reaction of S-nitration [16]. S-nitrosylation modifies the activity of some kinases and phosphatases, thus raising the possibility that NO modifies phosphorylation and dephosphorylation through S-nitrosylation.

NO reacts with oxygen, transitional metal ions, thiols, and superoxides, exerting its effects via cGMP-dependent and/or -independent pathways. cGMP effector molecules include cGMP-dependent protein kinases type-I and –II, cGMP-activated phosphodiesterases, and cGMP-gated ion channels. Similar to phosphorylation, S-nitrosylation regulates protein function allosterically or by direct modification of cysteine.

In the vascular system, NO reacts with sGC forming cGMP, which activates cGMP-dependent protein kinase decreasing vascular smooth muscle cell cytoplasmic Ca^{2+} concentration by 1) activation of proteins such as Ca^{2+}-sensitive potassium channels which decrease membrane potential thereby causing hyperpolarization and closing voltage dependent Ca^{2+} channels; 2) phosphorylation of voltage- and receptor-operated sarcolemmal Ca^{2+} channels, causing them to close; 3) inhibition of the inositol 1,3,5-trisphospate-sensitive Ca^{2+} release channel of the sarcoplasmic reticulum [17].

2.2. NO prevents the development of PH

NO mediates vasorelaxation, anticoagulation, and anti-proliferation, as well as neurotransmission. Several earlier studies demonstrated that NO inhibits smooth muscle cell growth by a cGMP-dependent mechanism [18] in addition to inhibiting growth regulating enzymes such as ribonucleotide reductase and thymidine kinase [19,20]. NO also suppresses the hypoxia-induced increase in ET-1 and platelet-derived growth factor-B, both of which have vasoconstriction and growth effects [21]. These effects of NO led investigators to determine whether administration of NO prevents the development of PH. Chronic NO inhalation ameliorates the development of hypertensive pulmonary vascular changes of chronic hypoxia-induced PH in rats [22], but not in monocrotaline (MCT)-induced PH [23]. In contrast, supplementation with the NO precursor, L-arginine, but not D-arginine prevented the development of PH in both models [24]. The reason for the different effects of NO inhalation is unclear, but may be a result of differing pathogenic mechanisms in the two models of PH: the increase in pulmo-

nary pressures precedes the vascular structural changes in chronic hypoxia-induced PH, whereas the reverse sequence of events occurs in MCT-induced PH. Endogenous NO from L-arginine could prevent the development of new muscularization of peripheral pulmonary arteries in both models, whereas exogenous inhaled NO would be effective only in hypoxia-induced PH because of the reduction in pulmonary vascular pressures caused by NO mediated vasodilation.

Inhaled NO likely attenuates the hypertensive vascular structural changes through pulmonary vasodilation by a cGMP-mediated mechanism. Endogenous NO from L-arginine might also prevent the development of structural changes through a cGMP-mediated mechanism. This hypothesis is supported by another study that showed that pulmonary gene transfection of atrial natriuretic peptide (ANP), another inducer of cGMP, attenuates the development of chronic hypoxia-induced pulmonary vascular changes [25]. Treatment to increase NO production in the pulmonary vascular bed by eNOS gene transfection ameliorates the development of PH. Studies have demonstrated that eNOS transfected smooth muscle cell administration prevented the development of MCT-induced PH [26] and that eNOS trans-fected bone marrow-derived endothelial-like progenitor cell venous administration reversed established MCT-induced PH [27].

3. Endogenous NO production

3.1. Nitrate (NO_3^-) and nitrite (NO_2^-) as sources of NO (Figure 1)

NO is produced from L-arginine by nitric oxide synthase (NOS) in the presence of oxygen, tetrahydrobiopterin (BH4), and reduced NADPH[3]. Recent studies have indicated that inorganic anions, nitrate (NO_3^-) and nitrite (NO_2^-), can be recycled to NO in vivo as alternative sources of NO in addition to the classical NOS-NO pathway. The source of nitrate includes the endogenous NOS-NO synthase pathway and the diet. Green vegetables such as lettuce and spinach provide nitrate and preservatives in cured meat and bacon include nitrite. Basically reduction of nitrate and nitrite produce NO, thus nitrate and nitrite are considered an 'endo-crine reservoir' of NO [28].

Nitrate in the plasma is excreted into the saliva, whereas nitrate is reduced by the oral anerobic bacteria producing nitrite. These bacteria use nitrate as an electron acceptor instead of oxygen during respiration. During its subsequent movement into the stomach, nitrite undergoes further reduction to NO, thus leading to gastric NO formation, which may play a role in gastric mucosa maintenance. This is a entro-salivary circulation of nitrate. In the systemic circulation intravascular nitrite is reduced to NO by deoxyHb, respiratory chain enzymes, xanthine oxidoreductase, deoxygenated myoglobin, and protons (29). They facilitate the transfer of protons to NO_2^-, causing NO production which is intensified under acidic and hypoxic states. Artery-to-vein gradients in nitrite are observed.

Nitrite has a vasodilatory effect. Inhaled nebulized sodium nitrite reduces pulmonary artery pressure (PAP) without changes in systemic artery pressure in hypoxia- or thromboxane-
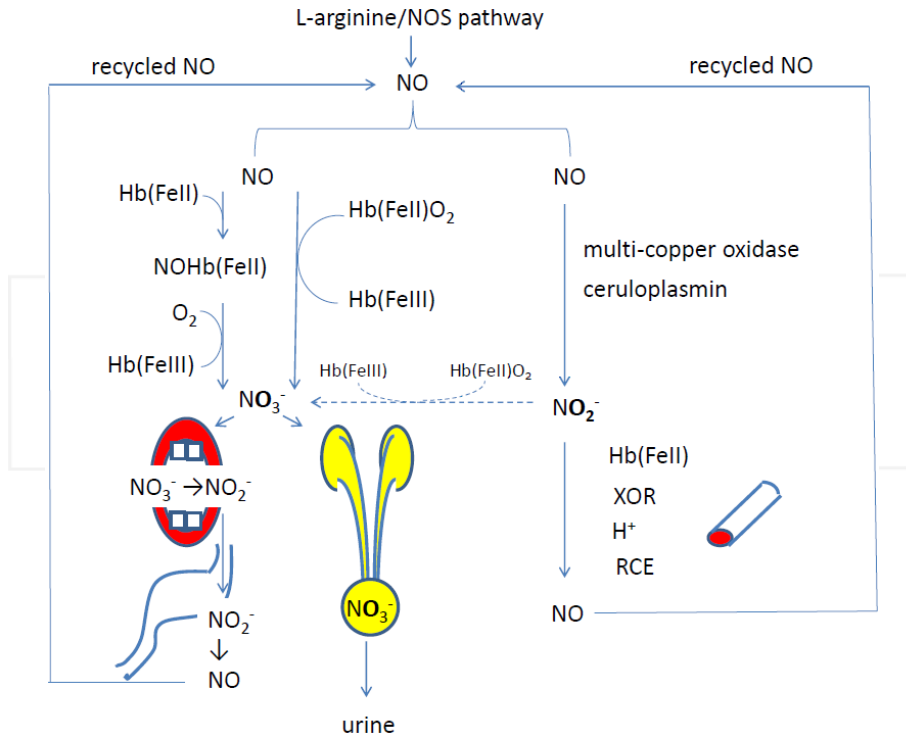
Figure 1. Recycling of NO from NO_2^- Endogenous NO includes NO produced from L-arginine by NOS and recycled NO from NO_2^-. NO is converted to NO_3^- by the reaction with the Hb and /or to NO_2^- by the oxidation in the plasma with the aid of multicopper oxidase and NO oxidase ceruloplasmin. NO_3^- is excreted into urine by kidney and/or into oral cavity by salivary gland. In the oral cavity anaerobic bacteria reduces NO_3^- converting to NO_2^-, which goes down into stomach and is protonated under the gastric acidic state forming nitrous acid (HNO_2) with further decomposition to NO and/or other nitrogen oxides. NO_2^- in the plasma is reduced and converted to NO by the reductase activity of deoxygenated hemoglobin, xanthine oxidoreductase, respiratory chain enzymes, and hydrogen ion. Hb(FeII), deoxygenated hemoglobin; Hb(FeII)O_2, oxygenated hemoglobin; NO, nitric oxide; NOHb(FeII), nitrosylhemoglobin; NOS, nitric oxide synthase; NO_2^-, nitrite, NO_3^-, nitrate; XOR, xanthine oxidoreductase; Hb(FeIII), methemoglobin; REC, respiratory chain enzymes

induced PH [30]. Intravenous administration of sodium nitrite reverses PH induced by hypoxia or thromboxane analogs [31]. Furthermore, intermittent nebulization of sodium nitrite ameliorated the muscularization and hyperplasia of small pulmonary arteries, the development of right ventricular hypertrophy, and the rise in right ventricular pressure in chronic hypoxia- or MCT-induced PH in rats [32], which is similar to L-arginine administration[33].

The effects of inhaled NO are not restricted to the lung. Recent studies have shown that inhaled NO improves neurological and left ventricular dysfunction after successful cardiopulmonary resuscitation [34] as well as liver function after liver transplantation [35]. Inhaled NO is converted to nitrate and nitrite when it enters the blood [36, 37]. NO can be recycled from nitrite and be used to protect organs from ischemia reperfusion injury.
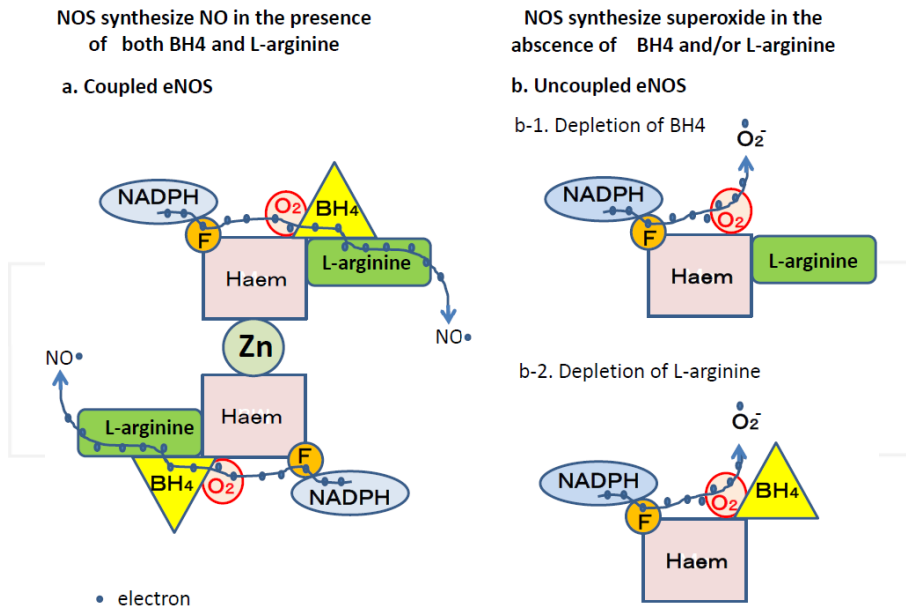
Figure 2. Coupled eNOS (eNOS homodimer) produces NO. (a) eNOS homodimer produces NO, whereas eNOS monomer produces superoxide. eNOS uncoupling occurs during the conversion of eNOS homodimer to eNOS monomer. Two eNOS monomers are connected with the aid of Zn^{2+}, making eNOS homodimer. BH4 strengthen the Zn^{2+} connection, maintaining the dimer form. In coupled NOS, an electron is transferred to L-arginine, producing NO and L-citrulline. (b) electron($+$) from NADPH is transferred to O_2 in the uncoupled eNOS in absence of BH4(b-1) and/or L-arginine(b-2), thereby producing superoxide. BH4, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; F, flavin; NADPH, nicotinamide adenine dinucleotide phosphate

3.2. NOS uncoupling: NOS produces NO and superoxide depending on whether it is a homodimer or monomer (Figure2)

In the process of NO formation from oxygen and L-arginine, oxygen molecules are incorporated in both NO and L-citrulline, showing that NOS is a dioxygenase [38]. NOS contains both a reductase domain and an oxygenase domain, where electron transfer occurs from the reductase domain to the oxygenase domain. NADPH and flavin bind to the reductase domain, while oxygen, BH4 and L-arginine bind to the oxgenase domain. Electrons are transferred from NADPH through the flavin containing reductase domain to the oxygenase domain [39]. Then two cascades of further electron transfer occur depending on the presence or absence of BH4 and L-arginine. When both BH4 and L-arginine are present, NO is synthesized by oxidative deamination of arginine by NOS, where the electron is transferred to L-arginine. The initial step of L-arginine oxidation is donation of electrons to the ferrous–dioxygen complex from BH4, where trihydrobiopterin is produced and the electron is supplied through flavin regaining BH4 [40]. In contrast, in the absence of L-arginine or BH4, NOS synthesizes the superoxide, where the electron is transferred to ferrous oxygen. Intracellular deficiency of BH4 induces superoxide generation from eNOS [40]. The term "eNOS uncoupling" means func-

tionally that electron transfer to L-arginine is uncoupled, when the electron is transferred to ferrous-dioxygen instead of L-arginine, producing superoxide. NOS homodimer produces NO from L-arginine and oxygen, whereas NOS monomer produces superoxide [41]. Thus, the molecular basis of eNOS uncoupling is conversion of the NOS dimer to the NOS monomer. To maintain the NOS dimer, BH4 is essential and dihydrobiopterin (BH2) is the oxidized form of BH4. Peroxinitrite oxidizes BH4 to BH2, reducing the BH4 amount and/or the BH2/BH4 ratio, both of which induce eNOS uncoupling [42]. The effects of BH4 are mediated through the regulation of NO compared with superoxide synthesis by endothelial NOS. Since BH4 might both augment NO synthesis and decrease superoxide production, BH4 deficiency may play a role in the pathogenesis of PH.

eNOS uncoupling is evaluated by the eNOS dimer/monomer ratio in cold SDS-PAGE Western blot analysis. While oxidative stress reduces the eNOS dimer/monomer ratio in a cardiac hypertrophic model suggesting eNOS uncoupling, exogenous BH4 restored the eNOS dimer/monomer ratio [43]. Administration of exogenous BH4 might be used for eNOS uncoupling diseases. BH4 deficiency might cause PH in mice and BH4 augmentation might ameliorate the development of PH. Mice with low BH4 tissue levels develop PH which is reversed by increasing BH4 with targeted transgenic overexpression of the rate-limiting enzyme in BH4 synthesis, guanosine triphosphate(GTP) cyclohydrolase [44]. Lung BH4 availability is controlled by pulmonary vascular tone, right ventricular hypertrophy, and vascular structural remodeling. BH4 is a cofactor of NOS in the production of NO. BH4 deficiency causes decreased NO production with concomitant production of superoxide by NOS. Chronic administration of BH4 analogues improves NO-mediated pulmonary artery dilatation in rats with chronic hypoxic pulmonary hypertension [45]. Copresence of increased levels of NOS and reduced NO bioactivity might be explained by the deficiency of BH4 and/or L-arginine.

Long-term increases in NO might increase eNOS expression and eNOS uncoupling, thereby producing superoxide. Long-term administration of nitroglycerin (TNG) increased eNOS mRNA and protein expression and vascular superoxide ($O_2^{\bullet-}$) in intact vessels monitored using ESR spectroscopy [46]. An earlier study showed that endothelial denudation improves vascular relaxation induced by TNG in isolated vessels from nitrate-tolerant animals [47].

3.3. Caveolin and NOS (Figure 3)

Caveolae are flask-shaped invaginations on the cell surface, which contain structural proteins called caveolin and other signaling proteins. In endothelial cells, eNOS is inactivated when it is conjugated to caveolin-1, a structural protein of endothelial caveolae; eNOS is activated when it dissociates from caveolae. Stimulation of β2 adrenergic receptors cause this dissociation through phosphorylation of Tyr in caveolin-1. The mouse pulmonary endothelial β2 adrenergic receptor coupled to Gi/o proteins causes phosphorylation of caveolon-1 by Src kinase and eNOS phosphorylation at ser[1177] by the Src kinase - phosphatidylinositol 3 kinase (PI3kinase) - Akt kinase pathway [48]. Thus, stimulation of the β2 adrenergic receptor causes endothelial NO synthase-dependent relaxation.

Loss of caveolin-1 induces chronic activation of eNOS and subsequent tyrosine nitration of PKG in lungs from patients with idiopathic pulmonary hypertension, where activated eNOS

is uncoupled eNOS, producing superoxide [49]. Genetic deletion of caveolin in mice causes PH and treatment with a superoxide scavenger and/or a NOS inhibitor prevents PH associated vascular remodeling [49]. Although caveolin expression in total lung determined by Western blotting is not altered in severe PH, its immunohistological expression in plexiform lesions is absent or decreased [50].

A 90-kDa heat shock protein (HSP90) is a molecular chaperone of proteins that modulates protein functions. Along with many other proteins, eNOS and sGC are targets for HSP90. HSP90 interacts with eNOS and HSP90 facilitates the displacement of eNOS from caveolin 1, activating eNOS. HSP90 activity is dependent on adenosine triphosphate (ATP). Asymmetric dimethylarginine (ADMA) inhibits HSP90 activity in pulmonary endothelial cells through mitochondrial dysfunction, caused by ADMA induced eNOS uncoupling with subsequent superoxide production and nitration of mitochondrial protein, which reduce ATP production [51].

3.4. eNOS expression and activity in PH

To examine whether the change in eNOS expression and its activity is associated with vascular endothelial dysfunction in PH, many studies have been performed in several species of animals and humans, using isolated lung, isolated pulmonary artery, and in vivo. eNOS is expressed in not only vascular endothelial cells, but lung epithelial cells. In addition, eNOS expression and/or activity might be different between conduit PAs and resistance PAs.

Animal models

mRNA and protein expression of eNOS in rat lung and eNOS expression localized in pulmonary vascular endothelial cells and epithelial cells is upregulated in acute hypoxia [52]. In that study, nitrate/nitrite in rat lung homogenate also increased, suggesting augmented eNOS activity. The enhancement of eNOS activity in hypoxic pulmonary vasoconstriction (HPV) in normal rat lung also has been shown in other studies using NOS inhibitors [53, 54] (see sect. 3.1). eNOS protein expression was time-dependently increased in rats in chronic hypoxia-induced PH [55,56], while phosphorylated eNOS (peNOS), active form, was impaired [55]. MCT-induced PH rats showed decreased expression of both eNOS [57,58,59] and peNOS [59].

Human

Many studies of eNOS expression and its activity have been performed in adult human PAH. However, the results are not consistent: eNOS expression is reduced in pulmonary vessels from adults with primary and secondary PH, but is increased in plexiform lesions [60]. Western blot analysis showed that eNOS expression is not changed in the lung tissue of idiopathic PAH (IPAH) patients [61]. However, several studies reported lower exhaled nitrate/nitrite (NOx) in PAH patients [62,63]. Overall, these results suggest that eNOS activity might be depressed in adult human PAH.
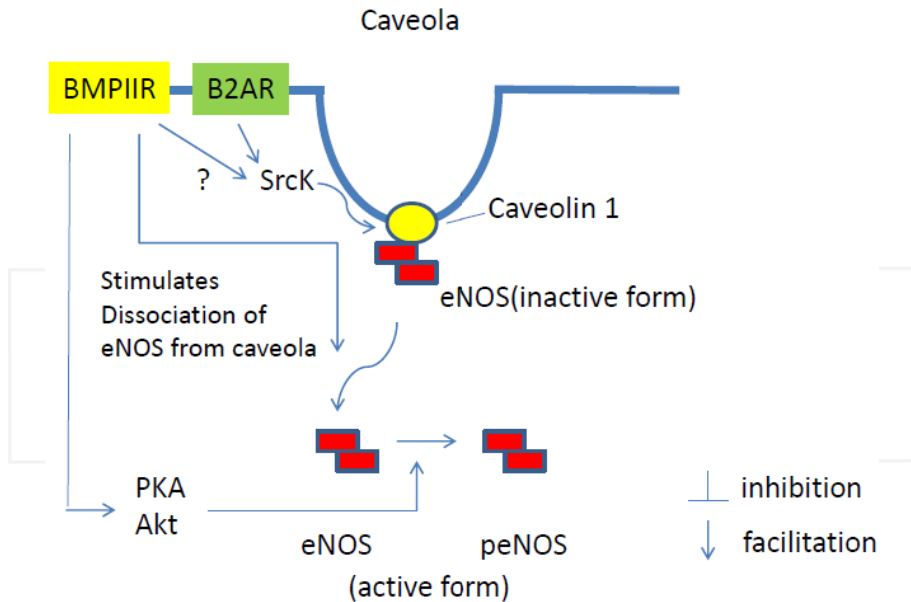
Figure 3. Inactive form of eNOS associated with caveola. eNOS is associated with caveola, which is the inactive form of eNOS. The active form of eNOS is dissociated from caveola. Stimulation of BMPIIR induces dissociation of eNOS from caveola as well as phosphorylation of eNOS through PKA and/or Akt activation. eNOS, endothelial nitric oxide synthase; B2-AR, beta 2-adrenergic receptor; SrcK, src kinase; peNOS, phosphorylated eNOS; BMPIIR, bone morphogenetic ptotein II receptor; PKA, cyclic AMP-dependent protein kinase

4. Endothelium-dependent and -independent NO-mediated relaxation in pulmonary circulation

4.1. Role of endothelium-derived NO in basal tone

L-NMMA (N omega-monomethyl–L-arginine), L-NNA (N omega-nitro-L-arginine), L-NAME (N omega-nitro-L-arginine methyl ester), L-NA(N omega-nitro-L-arginine) and other NOS inhibitors have been used to examine the physiological role of NO in pulmonary vascular tone. The increase in vascular tone in the presence of NOS inhibitors may indirectly represent NO production and/or release in the pulmonary circulation.

Animals

L-NMMA [53] and L-NNA [64,65] did not change pulmonary basal tone in normal rat PA rings. Normal isolated perfused lungs were not affected by NOS inhibitors such as L-NMMA [53], L-NNA [64], and L-NA [66] except for a few studies showing a moderate increase with L-NAME [67]. In chronic hypoxia, many studies showed markedly enhanced vascular tone by L-NNA [64] or L-NAME [67]. Although these NOS inhibitors caused different results, the findings suggested that 1) NO might not be involved in vascular basal tone in normal pulmonary circulation, and 2) basal NO production might be increased in hypoxia-induced chronic

PH. On exposure to acute hypoxia, NOS inhibitors augmented vascular contraction in normal [53,67,68] and hypoxia-induced PH rat models [67]. This finding suggests that NO production in HPV is increased in both normal and hypoxic PH rats.

Humans

Inhibition of NO production by L-NMMA caused the reduction of pulmonary flow in conscious healthy adults [69,70], suggesting the possible role of continuous production of NO in maintaining basal vascular tone. In PAH patients, several studies reported decreased expression of NOS. Although several studies reported decreased exhaled nitrogen oxide (NOx) levels in PAH patients, others have reported higher levels. The results therefore remain inconclusive.

4.2. Vasoreactivity to endothelium-dependent and independent NO-related relaxing substances in rat lung

Many studies have been performed using acetylcholine (Ach) and sodium nitroprusside (SNP), endothelium-dependent and -independent NO-related vasorelaxants, to examine functional changes in vascular endothelial and smooth muscle cells in PH. As Ach-induced relaxation was abolished by NOS inhibitors [64] and restored with L-arginine [71,72], reactivity may partly reflect changes in NOS expression and/or activity.

Rats with hypoxic PH

The relaxation response to Ach is impaired in rat isolated conduit pulmonary arteries (PAs) [65,73,74,75,76]. Many of these studies also described an impaired relaxation response to SNP in conduit PAs [65,74,76]. These results suggested 1) decreased production and release of NO in endothelial cells or 2) decreased responsiveness to NO in smooth muscle cells, or both. Impaired relaxation in Ach and SNP was partially restored after exposure to chronic hypoxia. As the recovery process was different between the responses of Ach and SNP [65], it was speculated that NO-related functional abnormalities in endothelial and smooth muscle cells occurred independently.

In contrast, in hypoxic vasoconstriction resistant rat PA rings, the relaxation response to Ach was not changed [74,75] or augmented [77] in chronic hypoxia. It is likely that Ach-reactive NO production and/or release varies in a vascular site-specific manner. Conduit arteries produce and release more eNOS than peripheral arteries. The vascular functional change in response to stimuli such as abnormal shear stress, circumferential wall stretch and hypoxia itself may occur in conduit PAs more than in peripheral resistant arteries. Although conduit arteries do not directly relate to pulmonary vascular resistance, the pathophysiological change in conduit arteries may play a key role in pulmonary vascular remodeling [78].

Impaired response to Ach was partly restored in the presence of a non-selective inhibitor of cyclooxygenase (COX) [65] or prostaglandin (PG) H_2 / thromboxane (TX) A_2 receptor antagonist [79], suggesting the possibility of 1) imbalance between the production of vasocontracting and vasorelaxing prostanoid in vascular endothelial cells, and 2) simultaneously release of vasocontracting prostanoids such as PGH_2 and/or TXA_2. Pidgeon et al. showed that the basal

expression of COX2, otherwise known as PGH synthase, was increased in rat lungs in chronic hypoxia, and a PGH_2/TXA_2 receptor antagonist attenuated the rise in PAP induced by chronic hypoxia [80].

MCT-induced PH in rats

PA vascular functional changes in rats with MCT-induced PH have been compared with PAs from animals with chronic hypoxia-induced PH. Many vasodilation studies have reported a depressed relaxation response to Ach in MCT-induced rat conduit PA rings [76,81,82,83,84]. Many of these studies described impaired SNP relaxation, [76,82] with the exception of one study [84]. While Ach-induced relaxation was impaired in the pulmonary circulation in MCT-induced PH, the SNP relaxation response has been reported to be impaired [85] or not impaired [86]. Taken together, in MCT-induced PH, vascular endothelial dysfunction is observed from proximal to distal PAs; however, smooth muscle functional alteration is not apparent in peripheral PAs.

5. Superoxide scavenges NO producing peroxynitrite (Figure 4)

5.1. Oxidative stress

In pulmonary hypertension, endothelial NOS expression is increased, which may not necessarily indicate an increase in NO production [87]. NOS might produce superoxide, which is due to uncoupling of NOS [88]. Increased levels of NOS and reduced NO bioactivity might be explained by the deficiency of BH4 and/or L-arginine. Oxidative stress induces the changes of BH4 to BH2. Oxidative stress also induces S-glutathionylation and subsequent eNOS uncoupling [39], in which S-glutathionylation of eNOS reversibly decreases NOS activity with an increase in $O_2^{\bullet-}$ generation primarily from the reductase and endothelium-dependent relaxation is impaired. Oxidative stress upregulates nuclear factor (NF)-kappaB, a key transcription factor that is involved in vascular tissue remodeling. NF-kappaB nuclear localization and vascular cell adhesion molecule 1(VCAM-1) expression is temporally and spatially associated with the development of MCT-induced PH in rats, which is ameliorated by administering a NF-kappaB inhibitor, pyrrolidine dithiocarbamate(PDTC)[89].

5.2. Production of superoxide in PH: role of NADPH oxidase and SOD

NAD(P)H oxidase enzyme complex catalyzes one electron reduction of oxygen using NADPH or NADH as an electron donor, which produces superoxide : $NAD(P)H + 2O_2 \rightarrow NAD(P)^+ + H^+ + 2O_2^-$ NADPH oxidase expression is increased in pulmonary arteries from a lamb model of persistent pulmonary hypertension of the newborn (PPHN) [90]. The expression was determined by the Western blotting of the levels of p67[phox] a subunit of the NADPH oxidase complex and immunostaining of the pulmonary vessels in lung sections. Another study demonstrated that expression and activity of the NADPH oxidase complex are upregulated in PH with increased pulmonary blood flow [91].
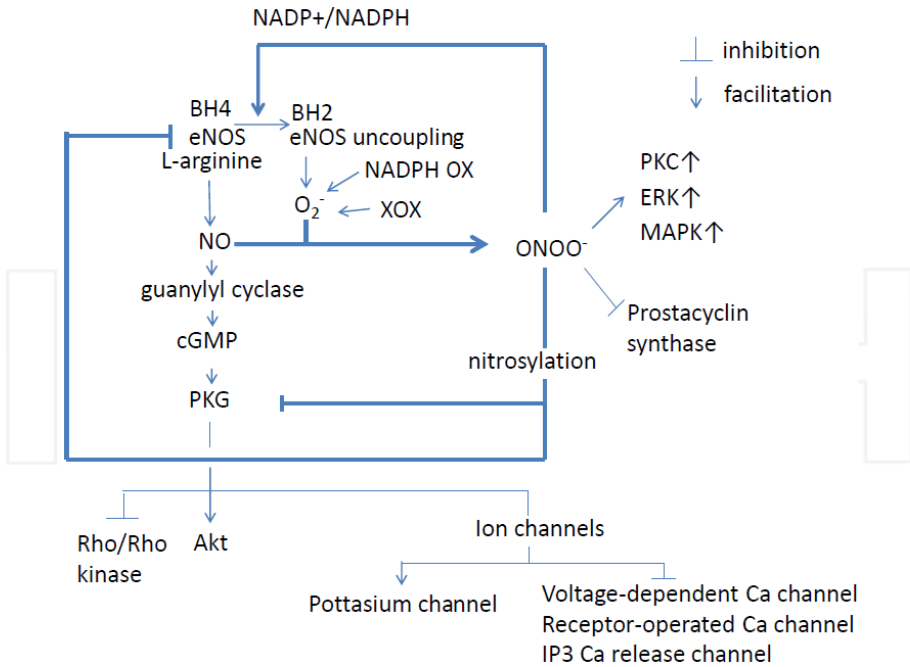
Figure 4. Peroxynitrite production from NO and superoxide. Superoxide (O_2^-) is produced by uncoupled eNOS, NADPH oxidase, and xanthine oxidase. NO reacts with O_2^- producing peroxynitrite($ONOO^-$) with subsequent nitrosylation of protein kinases, thereby activating or suppressing their activities. PKG phosphorylates Rho kinase, Akt, and ion channels. Phosphorylation of ion channels makes Ca^{2+} ion channels closed and potassium channel open. Peroxynitrite further oxidize BH4 to BH2, inducing eNOS uncoupling with subsequent superoxide production. BH4, tetrahydrobiopterin; BH2, dihydrobiopterin; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; IP3, inositol triphosphate receptor; MAPK, mitogen-activated protein kinase; OX, oxidase; $ONOO^-$, peroxynitrite; PKC, protein kinase C; PKG, cyclic-GMP dependent protein kinase(protein kinase G); XOX, xanthine oxidase;

Deficiency of superoxide dismutase (SOD) may play a role in the development of PH. Expression and activity of mitochondrial SOD2 in patients and animal models of PH is decreased [92,93] in pulmonary arteries and plexiform lesions. SOD produces H_2O_2 from mitochondrial superoxide. H_2O_2 is less potent than superoxide and acts as a signaling molecule to inhibit transcriptional factors such as hypoxia-inducible factor-1α. Epigenetic suppression of SOD with selective hypermethylation of CpG islands in SOD2 gene induces excessive proliferation and decreases apoptosis in pulmonary artery smooth muscle cells [92], suggesting a causative role of SOD deficiency in PH.

NO reacts with superoxide more rapidly than SOD producing peroxinitrite. Peroxynitrite is a more potent and versatile oxidant than NO or superoxide, in which HO^+ and NO_2 produced from peroxynitrous acid (HOONO) and/or its reactive activated isomer (HOONO*) attacks biological targets [94] including cyclic GMP-dependent protein kinase (PKG). In the setting of eNOS uncoupling, eNOS synthesizes superoxide which reacts with NO to create peroxynitrite. Nitrosylation of PKG by $ONOO^-$ depresses the function of PKG (42).

6. Prevention of hypertensive pulmonary vascular remodeling through NOS/NO pathway

6.1. NO precursor L-arginine ameliorates PH

Arginase, an enzyme in the urea cycle, converts arginine to ornithine and urea. NOx concentrations in exhaled gas and serum are decreased in PH patients compared with normal persons [95], suggesting decreased NO availability in PH. The deficiency of the NO precursor L-arginine, the substrate depletion of NOS, might partly explain the decrease in NO availability. Lower levels of arginine in the cell might be due to the increased activity of arginase. In PH patients, lower levels of arginine correlate with higher pulmonary artery pressures. Serum arginase activity is higher and the serum arginine-ornithine ratio is lower in PH patients than in healthy controls, indirectly suggesting increased intracellular arginase activity [33]. Animal studies showed that prolonged administration of L-arginine ameliorated the development of monocrotaine-induced PH [24,96] and chronic hypoxia-induced PH [96]. In patients with PH L-arginine treatment reduces PAP [97]. In addition to functioning as the substrate for NO formation, L-arginine prevents eNOS uncoupling, serves as a direct radical scavenger, and competes with the endogenous eNOS inhibitor ADMA, which decreases superoxide and increases NO formation [41].

6.2. ATRA increases NO production (Figure 5)

The level of asymmetrical dimethylarginine (ADMA) is increased in patients with PAH and MCT-induced PH in rats [98]. Since ADMA is an endogenous competitive inhibitor of NOS and suppresses NOS activity, increases in ADMA inhibit NO production. In atherosclerotic arteries from patients with high serum ADMA, endothelium-dependent relaxation by acetylcholine was impaired and $O_2^{\bullet-}$ production was increased [99]. Dysregulation of ADMA might cause PH through the decrease in NO in the lung as well. Dimethylarginine dimethyaminohydrolase (DDAH) is a metabolizing enzyme of ADMA. Thus the increase in DDAH activity reduces ADMA and induces subsequent increases in NOS activity. DDAH has two isoforms: DDAH 1 and DDAH 2. DDHA 1 and DDAH 2 are expressed predominantly in tissues containing neuronal NOS (nNOS) and eNOS, respectively [100]. Phosphodiesterase (PDE) 3/4 inhibitors reduce ADMA and raise NO/cGMP levels [2]; PDE3/4 inhibitors activate the cAMP/protein kinase A (PKA) pathway and induce subsequent activation of the promoter region of DDAH2. Western blot analysis of lung from PH rats 28 days after the injection of MCT showed decreases in eNOS, pNOS, AKT, and DDAH2 and increases in lung and serum ADMA levels [101]. In this PH model, 1) decreased Akt reduces eNOS phosphorylation and thereby decreases eNOS activity 2) decreased DDAH2 reduces ADMA breakdown and thereby the increase in ADMA inhibits eNOS activity. This study showed that rosuvastatin ameliorates MCT-induced PH through the normalization of Akt, eNOS and DDAH2 expression and ADMA levels [101].

Endothelial cells express retinoid receptors and all-trans-retinoic acid (ATRA) increased DDHA2 mRNA levels in endothelial cells. Although eNOS mRNA expression is not increased

with ATRA treatment, ATRA increases NO production, suggesting that ATRA increases activity of expressed eNOS indirectly through the decrease in ADMA due to increased DDHA2 [102]. ATRA also upregulates NO production in vascular endothelial cells through the PI3 kinase/Akt pathway [103]. ATRA induces eNOS phosphorylation at ser[1177] and Akt phosphorylation at ser[473] without changes in protein expression such as occur during DDAH2 upregulation. In terms of inducible NOS(iNOS), interleukin(IL)-1β increases iNOS mRNA levels and ATRA reduces this increase in vascular smooth muscle cell culture [104]. Because iNOS inhibition by the iNOS inhibitor N6-(1-iminoethyl-L-lysine, dihydrochloride(L-NIL) prevented the development of PH [105], the inhibitory effect of ATRA on iNOS expression might reduce the development of PH. Peroxisome proliferator-activated receptors (PPARγs) are a nuclear hormone receptor superfamily of ligand-activated transcription factors of retinoid hormone receptors other than steroid and thyroid hormones. PPARγ or retinoid X receptor (RXR) agonists inhibit smooth muscle proliferation. The PPARγ agonist rosiglitazone attenuates the development of chronic hypoxia-induced vascular structural remodeling [106], although it has little effect on the vasoconstriction component of PH. Since PPARγ mediates effects through the RXR, retinoids might also ameliorate PH vascular changes. PPARγ ligands increase the release of NO from endothelial cells through a transcriptional mechanism probably through the increase in DDAH mRNA expression without changes in eNOS expression [107]. These results suggest that ATRA might prevent the development of experimental PH in rats. ATRA ameliorated the development of MCT-induced PH [108], but not chronic hypoxia-induced PH [109]. These differences in the effect of ATRA on the development of PH may be due to a more pronounced inflammatory response in MCT-induced PH and a more subtle inflammatory reaction in chronic hypoxia-induced PH; endothelial damage precedes the rise in PAP in MCT model whereas the rise in PAP precedes endothelial changes in the chronic hypoxic model [110,111].

6.3. BMPIIR activates eNOS

Mutation of the bone morphogenetic protein receptor type II (BMPIIR) gene is one of the causes of familial PAH. The link between BMPIIR and eNOS partly explains the mechanism for the development of PH caused by BMPIIR mutations. Stimulation of BMPIIR induces eNOS phosphorylation, primarily through the cyclic-AMP dependent protein kinase and partially through serine-threonine kinase Akt [112]. Stimulation of BMPIIR also causes dissociation of eNOS from caveolin-1 and increases the eNOS-HSP90 interaction, which facilitates electron transfer through eNOS[112]. Thus, impaired BMPIIR or loss of BMPIIR stimulation might disturb the pulmonary vascular homeostasis, thereby causing PH.

6.4. VEGF increases eNOS expression

Vascular endothelial growth factor (VEGF) stimulates NO production initially by increasing intracellular Ca^{++} levels and subsequent Ca^{++}-calmodulin dependent activation of eNOS, and later by increasing intracellular eNOS message and protein levels [113]. VEGF stimulates vasodilation, microvascular hyperpermeability, and angiogenesis. Plexiform lesions show striking expression of VEGF associated with endothelial proliferation. NOS inhibition prevents
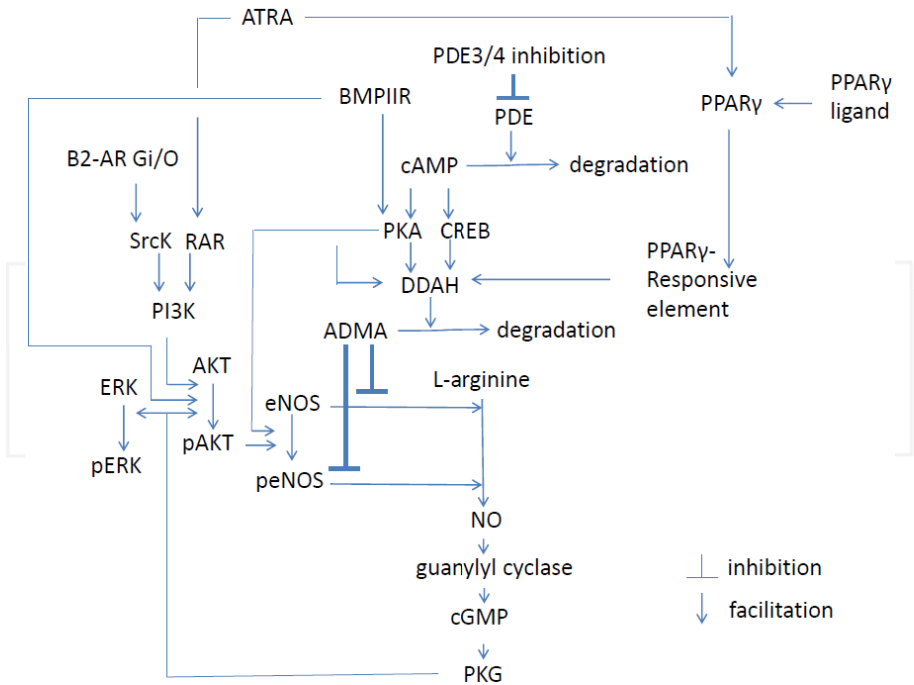
Figure 5. Possible pathway to enhance NO production by ATRA, DDAH, PDE3/4 inhibition, and BMPIIR. ADMA supresses NOS activity. DDAH is the enzyme that metabolizes ADMA. The cAMP/PKA pathway activates the promoter region of DDAH2, thereby increasing DDAH2 expression. ATRA increases DDAH2 mRNA, stimulates RAR with a subsequent increase in PI3K activity as well as PI3K protein and mRNA expression, and thereby enhances Akt and eNOS phosphorylation without increasing eNOS expression. Phosphorylated Akt(pAkt) phosphorylates eNOS making peNOS, the activated form of NOS. B2-AR stimulation activates SrcK via Gi/o protein. Activated SrcK phosphorylates PI3K and induces subsequent its downstream eNOS phosphorylation as well as phosphorylation of caveolin -1 to dissociate eNOS from caveola (Figure 3]. ADMA, asymmetric dimethylarginine; ATRA, all trans retinoic acid; B2-AR, beta 2-adrenergic receptor; BMPIIR, bone morphogenetic ptotein II receptor; CREB, cAMP responsive element binding protein; cAMP, cyclic adenosine monophosphate; DDAH, dimethylarginine dimethylaminohydrolase; eNOS, endothelial nitric oxide synthase; peNOS, phosphorylated eNOS; ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; Gi/o, GTP binding protein subunit Gi/o; PDE, phosphodiesteras; PI3K, phosphoinositide 3-kinase; PKA, cAMP dependent protein kinase; PKG, cyclic GMP-dependent protein kinase ; PPARγ, peroxisome proliferator-activated receptor; Srck, src kinase; RAR, retinoic acid receptor;

VEGF-induced proliferation in cultured microvascular endothelial cells, associated with the decrease in cGMP levels [114], suggesting that VEGF-induced proliferation is in part mediated by the NOS-NO-cGMP pathway. VEGF induces translocation of eNOS and caveolin-1 from caveola to the nucleus, where NO production activates transcriptional factors thereby inducing the early growth response gene, c-fos [115] and possibly inducing angiogenesis, and endothelial cell growth. VEGF receptor 2 (VEGF2R) blockade combined with chronic hypoxic exposure causes PH with plexiform like lesions, where decreased expression of VEGF2R, Src, Akt, phosphorylated Akt protein in lung have been demonstrated [116]. Studies have demonstrated that reduced Src and Akt attenuate eNOS phosphorylation [101].

6.5. Elastase inhibition by NO

Earlier studies have shown that vascular elastase activity is increased in MCT-induced PH and chronic hypoxia-induced PH in rats [3,117], and that elastase inhibition prevents the development of pulmonary hypertension, right ventricular hypertrophy, muscularization of peripheral pulmonary arteries and medial hypertrophy of muscular arteries [3,117,118]. NO might reduce the elastase activity through its scavenging effect of superoxide. Reactive oxygen species inactivates endogenous elastase inhibitor, α1-protease inhibitor, and might increase elastase activity [119]. Furthermore NO might reduce elastase expression by inhibiting its transcriptional factor, acute myeloid leukemia factor 1 (AML-1), through extracellular signal-regulated kinase mitogen-activated protein kinase (ERK MAPK) inhibition which is mediated by cGMP dependent protein kinase activation [120].
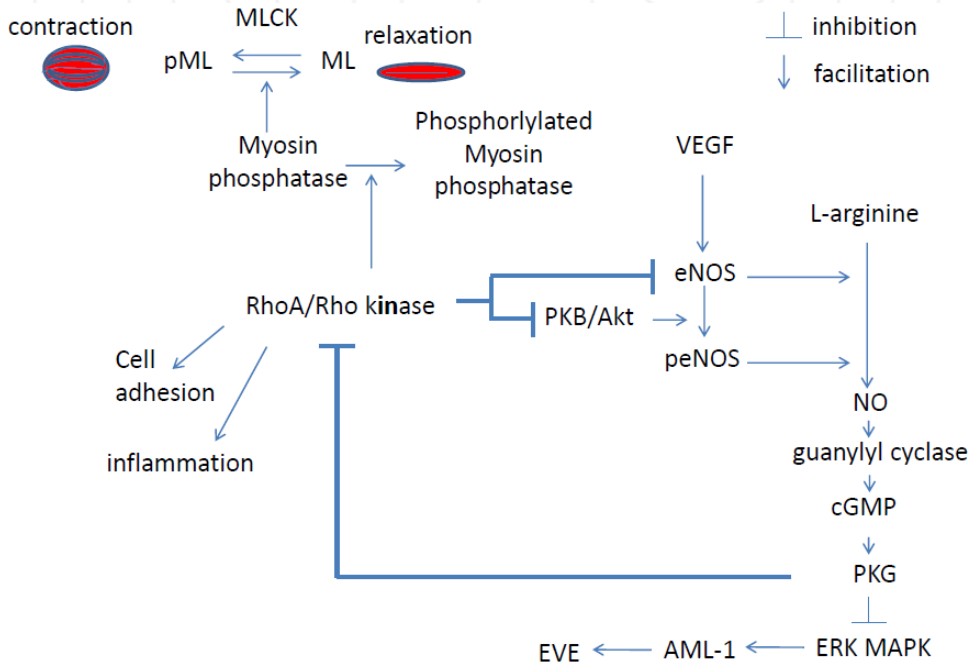

Figure 6. Rho/Rho kinase pathway inhibits eNOS/NO/cGMP pathway Rho kinase is activated by the guanosine triphosphate (GTP)-bound, active form of RhoA (GTP RhoA). Activated Rho kinase phosphorylates and subsequently inactivates myosin phosphatase, causing smooth muscle contraction, which is the RhoA/Rho kinase pathway. PKG phosphorylates Rho A at Ser[188] and inhibits Rho A function, thereby inactivating the RhoA/Rho kinase pathway. Activated RhoA/Rho kinase decreases eNOS mRNA and protein expression, inactivates Akt, and inhibits PKG activity, thereby supressing the eNOS/ NO/cGMP pathway. VEGF upregulates eNOS mRNA and protein expression. AML-1, acute myeloid leukemia factor 1(transcriptional factor); EVE, endogenous vascular elastase; PKG, cyclic GMP-dependent protein kinase(G kinase); PKB, protein kinase B(=Akt), AML-1, acute myeloid leukemia factor 1; ML, myosin light chain; pML, phosphorylated myosin light chain; MLCK, myosin light chain kinase; ERK MAPK, extracellular signal-regulated kinase mitogen activated protein kinase; VEGF, vascular endothelial growth factor.

6.6. Rho-kinase inhibitor upregulates NOS in PH (Figure 6)

Myosin light chain (MLC) phosphorylation by myosin light chain kinase (MLCK) causes vascular smooth muscle contraction. In contrast, myosin light chain dephosphorylation by myosin light chain phosphatase causes relaxation. The phosphorylation status of MLC phosphatase determines the contractility of smooth muscle at the same Ca^{++} concentration, thereby regulating the Ca^{++} sensitivity for contraction; the stronger the phosphatase activity, the weaker the vascular tone at the same Ca^{++} concentration. RhoA/Rho-kinase activation augments the phosphorylation of MLC phosphatase, which results in inhibition of MLC phosphatase. Studies have shown that Rho-kinase in circulating neutrophils is increased in patients with PH and that Rho-kinase expression is upregulated in isolated lung tissue on transplantation [121]. Rho-kinase activity in pulmonary arteries is enhanced in experimental PH [122,123]. NO-cGMP-cGMP dependent protein kinase pathway suppresses Rho/Rho kinase activity [124]. On the other hand Rho/Rho-kinase activation downregulates eNOS expression and eNOS phosphorylation through the inhibition of the protein kinase B/Akt pathway [125].

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