

## Review Article

# Pulmonary Hypertension Is a Probable NO/ONOO<sup>-</sup> Cycle Disease: A Review

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The NO/ONOO<sup>-</sup> cycle is a primarily local biochemical/physiological vicious cycle that appears to cause a series of chronic inflammatory diseases. This paper focuses on whether the cycle causes pulmonary arterial hypertension (PAH) when located in the pulmonary arteries. The cycle involves 12 elements, including superoxide, peroxynitrite (ONOO<sup>-</sup>), nitric oxide (NO), oxidative stress, NF- $\kappa$ B, inflammatory cytokines, iNOS, mitochondrial dysfunction, intracellular calcium, tetrahydrobiopterin depletion, NMDA activity, and TRP receptor activity. 10 of the 12 are elevated in PAH (NMDA?, NO?) and 11 have documented causal roles in PAH. Each stressor that initiates cases of PAH acts to raise cycle elements, and may, therefore, initiate the cycle in this way. PAH involves a primarily local mechanism as required by the cycle and the symptoms and signs of PAH are generated by elements of the cycle. Endothelin-1, which acts as a causal factor in PAH, acts as part of the cycle; its synthesis is stimulated by cycle elements, and it, in turn, increases each element of the cycle. This extraordinary fit to the principles of the NO/ONOO<sup>-</sup> cycle allows one to conclude that PAH is a NO/ONOO<sup>-</sup> cycle disease, and this fit supports the cycle as a major paradigm of chronic inflammatory disease.

## 1. Introduction

Pulmonary arterial hypertension (PAH) is a progressive and often fatal disease characterized by several important properties including inflammation, oxidative/nitrosative stress, and mitochondrial dysfunction. Such hypertension leads to right ventricular dysfunction that leads in turn to subsequent right heart failure and death. A number of other chronic diseases that share properties described in the first sentence, above, are thought to be caused by a primarily local biochemical vicious cycle, known as the NO/ONOO<sup>-</sup> cycle (pronounced no, oh no!) [1–11]. Thus the hypothesis being explored in this paper is whether pulmonary hypertension is caused by the local action of the NO/ONOO<sup>-</sup> cycle in the pulmonary arteries.

One of the testable properties of NO/ONOO<sup>-</sup> cycle diseases is that for the cycle to be causal, the symptoms and signs of the disease must be generated by elements of the cycle. The classic properties of PAH are vasoconstriction and pulmonary fibrosis and arterial remodeling. Hypertension can be generated by excessive peroxynitrite (ONOO<sup>-</sup>), which

is a vasoconstrictor [12–16], acting in part via oxidative stress and consequent elevated isoprostanes, because isoprostanes are potent vasoconstrictors [12, 13]. Elevated ONOO<sup>-</sup> can lead to oxidation of tetrahydrobiopterin (BH<sub>4</sub>), which may lead, in turn, to what is called the partial uncoupling of the nitric oxide synthases (NOSs), leading in turn to chronic ONOO<sup>-</sup> elevation and, in some cases, chronic hypertension [14–16]. All of these changes, discussed earlier in this paragraph, are thought to be important consequences of the NO/ONOO<sup>-</sup> cycle and are also thought to be involved in PAH [17, 18]. The properties of peroxynitrite (ONOO<sup>-</sup>) itself are quite distinct from those of its nitric oxide (NO) precursor, because NO is, of course, a vasodilator.

Pulmonary fibrosis and arterial remodeling are thought to be caused by oxidative stress, inflammatory biochemistry, and mitochondrial dysfunction in the pulmonary arteries [19–22], leading, in turn, to increased hypertension. Because all three of these causes are parts of the NO/ONOO<sup>-</sup> cycle, the cycle can, in these ways, produce the fibrosis and tissue remodeling that are critical hallmarks of the disease. Consequently both the vasoconstriction and the local fibrosis

and remodeling can be understood as being caused, at least in part, by four elements of the NO/ONOO<sup>-</sup> cycle, elevated ONOO<sup>-</sup> (previous paragraph), consequent oxidative stress, inflammatory responses and mitochondrial dysfunction.

One of the other testable properties of NO/ONOO<sup>-</sup> cycle diseases is produced by the primarily local nature of the cycle. Thus, the cycle predicts that if PAH is a NO/ONOO<sup>-</sup> cycle disease, it will be caused by local action of the cycle in the pulmonary arteries. One type of evidence that strongly supports such a local mechanism is the response of the disease to lung transplantation [23, 24]. The local nature of the fibrosis and remodeling, discussed in the previous paragraph, also supports a local mechanism, as do various other types of histological studies, showing local changes [25–28].

It can be seen from this discussion, that pulmonary hypertension appears to be in good agreement with these two predictions of the NO/ONOO<sup>-</sup> cycle, suggesting that it may be a NO/ONOO<sup>-</sup> cycle disease. In order to look at other predictions, we need to consider the properties of the cycle and how well they fit other properties of pulmonary hypertension.

## 2. Basic Properties of the NO/ONOO<sup>-</sup> Cycle

The latest version of the NO/ONOO<sup>-</sup> cycle is diagrammed in Figures 1(a)–1(e) (discussion taken from the author's web site with permission). Each of the arrows in Figure 1 represents one or more mechanisms by which one element of the cycle can stimulate a second element (see [1–3, 8, 10] for further detailed discussion). Near the core of the cycle (center, slightly left), nitric oxide (NO) reacts with another free radical, superoxide (·OO<sup>-</sup>) to form ONOO<sup>-</sup>, a potent nonradical oxidant.

Figures 1(a) through 1(e) differ from one another in that each of them diagrams how groups of different mechanisms of the NO/ONOO<sup>-</sup> cycle forms complete and in most cases multiple cycles which will act to propagate themselves over time, as is the nature of all vicious cycles. Thus without knowing anything about the elements of the cycle, one can see that if these diagrams are correct, each of these parts of the overall cycle (Figures 1(a) through 1(e)) will tend to interact with each other through their common elements to form a robust and difficult to downregulate compound cycle that we call the NO/ONOO<sup>-</sup> cycle. (Note: much documentation for this section is provided in the next section, which focuses on the specific mechanisms of these arrows).

Let us consider dashed arrows in Figure 1(a) starting again from the reaction of NO with superoxide to form peroxynitrite (ONOO<sup>-</sup>). Elevated ONOO<sup>-</sup> produces oxidative stress, an imbalance between oxidants and antioxidants. Both ONOO<sup>-</sup> and oxidative stress activate the transcription factor NF-κB (lower right) which activates, in turn, the transcription of both the inducible nitric oxide synthase gene (iNOS) and also several inflammatory cytokines (box, upper right). Each of these cytokines is linked to NF-κB by a double-headed arrow, such that each of them has its synthesis stimulated by NF-κB and most also, in turn, increase NF-κB

activity and some of them can also increase iNOS induction independently of NF-κB. Some of the cytokines can also act independently of NF-κB to increase iNOS activity. Each of these activities, then, can produce increases in iNOS activity, leading, in turn, to increased NO, thus producing a complete cycle.

There are also at least four other major cycles that are each parts of the overall NO/ONOO<sup>-</sup> cycle. The simplest of these is what is called the central couplet, the reciprocal elevation of ONOO<sup>-</sup> and depletion of tetrahydrobiopterin (BH4), (slightly below and right of center, Figure 1(c)) [1G, 1I]. ONOO<sup>-</sup> is known to oxidize and therefore deplete BH4 and BH4 depletion is known to produce a partial uncoupling of the NO synthases (eNOS, nNOS, and iNOS). When these NOSs are uncoupled, they produce superoxide in place of NO. Because the reaction of these two compounds is extremely rapid, but there are mechanisms which lead to rapid loss or sequestration of them in the cell, the synthesis of both on nearby enzymes is expected to be particularly efficient in producing ONOO<sup>-</sup>, a potent oxidant. Thus, ONOO<sup>-</sup> will produce BH4 depletion which will be expected to produce more ONOO<sup>-</sup>. This central couplet is thought to be particularly important in switching on the cycle [8], because NO acts to lower both NF-κB activity and NMDA activity, both important parts of the NO/ONOO<sup>-</sup> cycle. It can be argued, therefore, that decreasing the ratio of NO to ONOO<sup>-</sup> may be required to produce a chronic cycle and consequent chronic disease. This central couplet, as discussed below, appears to be particularly important to our understanding of pulmonary hypertension.

Other parts of the cycle (see Figure 1(b)) involve a very complex series of events, both intramitochondrial and also extramitochondrial, leading to mitochondrial dysfunction and consequent ATP depletion (lower, left). The intramitochondrial sequence is often initiated by NO, but involves superoxide, ONOO<sup>-</sup>, inactivation of mitochondrial proteins, and oxidation of the cardiolipin in the inner membrane in the mitochondrion. The extramitochondrial sequence is triggered by ONOO<sup>-</sup>, leading to major stimulation of poly (ADP-ribose) polymerase (often designated PARP or PARS), leading to the depletion of the enzyme substrate NAD and consequently also its reduced form, NADH. Depletion of NADH, because it is the most important source of hydrogen reductants entering the mitochondrion, will lead to mitochondrial dysfunction and ATP depletion. Lowered energy metabolism is known to act via two mechanisms to increase activity of the NMDA receptors (Figure 1(b), top center) which acts in turn to increase levels of intracellular calcium and consequent eNOS and nNOS activity (these both being calcium-dependent enzymes), leading to increased NO and ONOO<sup>-</sup>, feeding back into the mitochondrial cascade and ATP depletion.

An additional cycle (Figure 1(d)) includes three of the TRP group of receptors (upper left) which are known to be stimulated by oxidative stress (TRPA1, TRPV1, and TRPM2); these and other members of this receptor group are also reported to be stimulated by NO. The NMDA receptors, glutamate receptors involved in producing excitotoxicity act, as do the TRP receptors to increase intracellular calcium levels,

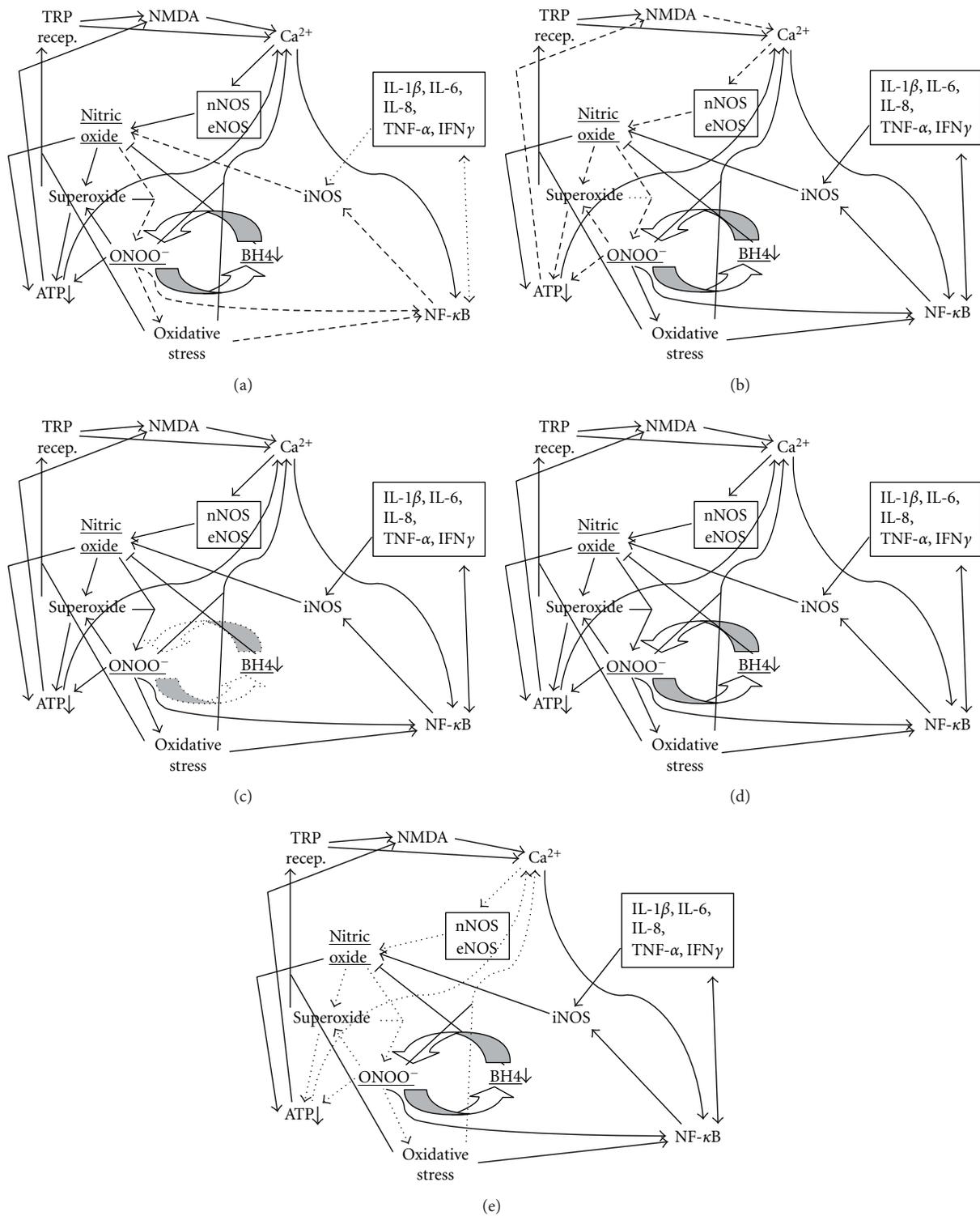


FIGURE 1: 1(a)–1(e) are essentially identical diagrams of the proposed NO/ONOO<sup>-</sup> cycle, where each arrow represents one or more mechanisms whereby one element of the cycle acts to increase the levels of a second element of the cycle. Each of these differs from the others in that arrows involved in cycles that constitute parts of the overall NO/ONOO<sup>-</sup> cycle are dashed, so that these constituent cycles can be considered independently of each other.

which act, in turn, to stimulate two of the calcium-dependent NOSs, eNOS, and nNOS, leading back to increased NO, superoxide, ONOO<sup>-</sup>, and oxidative stress, leading in turn to increased activity of some of these TRP receptors.

Figure 1(e) is focused on the properties of the plasma membrane calcium ATPase, which acts to pump excessive intracellular calcium out of the cell, an enzyme which is inactivated by both ONOO<sup>-</sup> and other oxidants and being an ATPase, its activity will be, of course, lowered by lowered energy metabolism. All of these interact with each other (Figure 1(e)) to form another complex vicious cycle.

Important, testable predictions of the overall NO/ONOO<sup>-</sup> cycle are discussed in the second section, below.

### 3. 34 Specific NO/ONOO<sup>-</sup> Cycle Mechanisms

What has become known as the NO/ONOO<sup>-</sup> cycle has become increasingly complex over time, as it has become clear that additional mechanisms should be considered as integral parts of the cycle. The current list of cycle mechanisms is as follows.

- (1) Extremely rapid diffusion-limited reaction between nitric oxide (NO) with superoxide (OO<sup>-</sup>), forming peroxynitrite (ONOO<sup>-</sup>) [1–3, 5, 29–31].
- (2) ONOO<sup>-</sup>, a potent oxidant, can act to increase the activity of the transcription factor NF-κB [5, 32–34].
- (3) ONOO<sup>-</sup> breaks down both before and after reaction with carbon dioxide into the following free radicals, hydroxyl (HO), carbonate (CO<sub>3</sub>), and NO<sub>2</sub> radical (NO<sub>2</sub>), each of which are responsible for a number of consequences produced by ONOO<sup>-</sup> [1–3, 35, 36].
- (4) ONOO<sup>-</sup> being a potent oxidant produces oxidative stress, an imbalance between oxidants and antioxidants [1–3, 30, 31, 35, 36].
- (5) Oxidative stress also produces increases in NF-κB activity because its activity is stimulated by oxidants and inhibited by chain-breaking antioxidants [2, 32–34, 37, 38].
- (6) NF-κB produces increased transcription of the inducible nitric oxide synthase (iNOS), a gene whose transcription is known to be stimulated by NF-κB elevation [1, 5, 33, 34] and whose elevation also stimulates much of the inflammatory cascade [39].
- (7) NF-κB also stimulates the transcription of several inflammatory cytokines, including IL-1β, IL-6, IL-8, TNF-α, and IFNγ [1, 5].
- (8) Each of the cytokines listed in 7 above, act directly and/or indirectly to stimulate the transcription of the iNOS gene, acting in some cytokines via the double-headed arrow linking them to NF-κB and, also, acting in some cytokines directly on iNOS induction [1, 5, 37–42].
- (9) When iNOS is induced, it produces large amounts of NO.
- (10) ONOO<sup>-</sup> inactivates the plasma membrane calcium-ATPase, leading to lowered calcium extrusion and increased levels of intracellular calcium [1, 43].
- (11) Other oxidants inactivate the plasma membrane calcium-ATPase, leading to increased levels of intracellular calcium [44–48]; such inactivation of the calcium ATPase has substantial pathophysiological effects [45–48] and may well contribute to the prolonged impairment of calcium extrusion seen under circumstances, where the NO/ONOO<sup>-</sup> cycle may have a role [49–51].
- (12) Lowered energy metabolism (decreased energy charge/ATP) also lowers calcium-ATPase activity, leading to increased levels of intracellular calcium [52], as predicted for such an ATPase.
- (13) While modest elevation of mitochondrial calcium, leads to increased ATP synthesis, substantial elevation of intracellular calcium leads to substantial increases in intramitochondrial calcium, leading to increased superoxide generation in the mitochondrion [49–51, 53]; large increases in mitochondrial calcium will lead, in some circumstances, to apoptotic cell death [50, 51, 53].
- (14) Intracellular calcium stimulates the nNOS and eNOS forms of nitric oxide synthase, both of which are calcium-dependent enzymes.
- (15) Increased nNOS and eNOS activity both produce increased NO synthesis.
- (16) ONOO<sup>-</sup> oxidizes tetrahydrobiopterin (BH<sub>4</sub>), depleting BH<sub>4</sub> levels [1, 2, 8, 10].
- (17) BH<sub>4</sub> depletion produces partial uncoupling of the three NO synthases, such that some of these enzymes produce superoxide in place of NO. Because of the very rapid reaction of these two compounds to produce ONOO<sup>-</sup>, this partial uncoupling involving nearby NOS enzymes is expected to produce an increase in ONOO<sup>-</sup> production [8, 10].
- (18) Nicking of nuclear DNA by ONOO<sup>-</sup> and hydroxyl and other radicals can produce a massive stimulation of poly ADP-ribose polymerase (PARP) and consequent poly-ADP ribosylation of chromosomal proteins, leading, in turn, to a massive depletion of NAD/NADH pools, because NAD is the substrate for such poly-ADP-ribosylation [1, 2, 54]. NADH depletion lowers, in turn, ATP production in the mitochondrion.
- (19) Other changes causing ATP depletion come from a cascade of events occurring within the mitochondrion. The cascade starts with NO, possibly produced by mitochondrial NO synthase (mtNOS which is thought to be largely a form of nNOS), with NO binding to cytochrome oxidase, competitively inhibiting the ability of molecular oxygen to bind. This inhibits the ability of cytochrome oxidase to serve as the terminal oxidase of the mitochondrial electron transport chain [1, 2, 55–58].

- (20) The action of NO, in 19 above, produces increased superoxide production by the electron transport chain [1, 2, 56–58].
- (21) ONOO<sup>-</sup> in the mitochondrion also acts to produce increased superoxide from the electron transport chain [1, 2, 56, 58].
- (22) Peroxynitrite (ONOO<sup>-</sup>), superoxide, and their products lead to lipid peroxidation of the cardiolipin in the inner membrane of the mitochondrion. Cardiolipin is highly susceptible to such peroxidation, because most of the fatty acids that make up its structure in mammals are polyunsaturated fatty acids, which are much more susceptible to peroxidation than are other fatty acids [1, 2, 10, 59–62].
- (23) Cardiolipin peroxidation leads to lowered activity of some of the enzymes in the electron transport chain, leading to further lowering of ATP synthesis [59–62].
- (24) Cardiolipin peroxidation also leads to increased superoxide generation from the electron transport chain in the mitochondrion [59, 62].
- (25) ONOO<sup>-</sup> produces inactivation of the mitochondrial superoxide dismutase (Mn-SOD) as well as the copper-zinc superoxide dismutase, leading in turn to increased superoxide levels [1, 2, 63–65].
- (26) ONOO<sup>-</sup>, superoxide, and NO all inactivate or inhibit the aconitase enzyme, lowering citric acid cycle activity and subsequent ATP synthesis [1, 5, 66].
- (27) Oxidative stress leads to oxidation of cysteine residues in the enzyme xanthine reductase, converting it into xanthine oxidase which produces superoxide as a product, thus increasing superoxide generation [1, 67].
- (28) Increased activity of the enzyme NADPH oxidase, which produces superoxide as a product, is an important part of the inflammatory cascade and contributes, therefore, to the cascade by producing increased superoxide [68, 69].
- (29) Activation of the NMDA receptors allows calcium influx into the cell, raising intracellular calcium levels including mitochondrial calcium levels [1, 2, 9, 49, 51, 53].
- (30) Activity of transfer receptor potential (TRP) receptors also allows calcium influx into the cell, again raising intracellular calcium levels [1, 2], presumably leading to increased nitric oxide production.
- (31) The main physiological agonist of the NMDA receptors is glutamate whose extracellular concentration is lowered after release by energy-dependent transport. It follows that ATP depletion produces increased NMDA stimulation by lowering glutamate transport [1, 2].
- (32) The activity of the NMDA receptors is also greatly increased by ATP depletion within the cells containing these receptors. The mechanism here is that the ATP depletion produces partial depolarization

of the plasma membrane, which produces, in turn, increased susceptibility of the NMDA receptors to stimulation [1, 2, 9].

- (33) Three of the TRP group of receptors have been shown to be stimulated by increased superoxide and/or oxidative stress or their downstream consequences, these being the TRPV1, TRPA1, and TRPM2 receptors, with the increased TRPV1, and TRPA1 activity being produced in part through the oxidation of cysteine residue side chains [70–74]. Several TRP receptors are also activated by nitric oxide-mediated nitrosylation [75].
- (34) TRPV1, TRPA1, and probably several other TRP group receptors, receptor stimulation has each been repeatedly shown to lead to increased NMDA activity [76–96], with neurons containing these TRP family of receptors acting in part by releasing glutamate, the major physiological NMDA agonist.

We have, in summary, 34 distinct, well-documented biochemical/physiological mechanisms that make up the complex vicious cycle we call the NO/ONOO<sup>-</sup> cycle. Most if not all of these are well-accepted biochemistry and physiology and most if not all of these 34 have been shown to play pathophysiological roles in one or more diseases. Consequently, there is little that is new regarding the cycle, except that when the individual mechanisms are put into juxtaposition with each other, they constitute a series of interacting cycles (Figure 1) which, based on their interactions, are likely constitute a robust vicious cycle, the NO/ONOO<sup>-</sup> cycle, which, is likely to be a major challenge to effectively downregulate.

#### 4. Is Pulmonary Hypertension a NO/ONOO<sup>-</sup> Cycle Disease? Other Predictions of the Cycle Mechanism

There are five principles that underlie the NO/ONOO<sup>-</sup> cycle, each of which makes predictions that can be used to determine if a specific disease is a good candidate to be caused by the NO/ONOO<sup>-</sup> cycle.

- (1) Short-term stressors that initiate the disease should be able to act by raising cycle elements.
- (2) The various elements of the cycle, with the possible exception of NO [8], should be elevated in the chronic phase of the disease.
- (3) The symptoms and signs of the disease should be produced by one or more elements of the cycle.
- (4) The basic mechanism of the cycle is local and such that it is localized to different tissues in different individuals. The reason for this primarily local nature is that the three inorganic compounds involved, NO, superoxide, and ONOO<sup>-</sup>, have limited half-lives in biological tissues. And the mechanisms of the cycle, those various arrows, act at the level of individual cells. This allows for great variations in tissue distribution from one patient to another, producing a huge

spectrum of illness. The point here is not that there are no systemic changes—clearly antioxidant depletion, neuroendocrine, and immune system changes—and the actions of some inflammatory cytokines will be to some extent systemic. But rather this primarily local nature gives much inherent variation due to the varying tissue localization of the basic mechanism (see Chapter 4, in [1]). A correlate of the primarily local nature of the cycle is that different NO/ONOO<sup>-</sup> cycle diseases will differ from one another in what tissue or tissues must be impacted by the cycle in order to be diagnosed as a specific cycle-caused disease.

- (5) Treatment of the disease should involve using agents that lower various parts of the cycle. In other words, we should treat the cause of the disease, not the symptoms.

Evidence has already been provided in the introduction, showing that pulmonary hypertension has a good fit to principles 3 and 4. That is, the symptoms and signs of PAH can be generated by elements of the cycle. In addition, the primarily local nature of the disease has been demonstrated by three different types of observations.

Let us consider the fit to the other three principles.

## 5. Principle 1

Principle 1 states that if PAH is a NO/ONOO<sup>-</sup> cycle disease, there should be plausible mechanisms by which stressors that initiate cases of the disease can raise NO/ONOO<sup>-</sup> cycle elements and thus can, at least in principle, initiate cases of the disease by initiating the cycle. While idiopathic cases of PAH have no identified stressor, other types of cases do have such stressors. In contrast to other proposed NO/ONOO<sup>-</sup> cycle diseases, it should be noted that the stressors implicated in PAH are often chronic stressors rather than short term stressors.

*High altitude PAH* is a well-established problem in areas of the world where people live at high altitude, notably in regions of central Asia and in the Andes mountains of South America [97–99]. PAH is thought to be triggered, in this condition, by *hypoxia*. Although animals are susceptible to high altitude PAH [100, 101], most animal model studies of this condition have studied animal responses to hypoxia [102–108].

Kho0 et al. [102] studied a mouse model of PAH caused by a mutation that greatly lowers the synthesis of BH4, another cycle element, which causes a greatly increased susceptibility of hypoxia-induced hypertension. A role of BH4 depletion is also suggested by studies showing that Tibetans are genetically resistant to high-altitude PAH and that they also have higher levels of NO exhaled from their lungs. This suggests that Tibetans may have higher levels of BH4, allowing them to synthesize more NO by increasing the coupling of the NOSs to BH4. However, because BH4 levels have not been measured in Tibetans, this interpretation must be viewed as an untested hypothesis.

Other studies implicate NO/ONOO<sup>-</sup> cycle elements in hypoxic PAH. Fantozzi et al. [103] showed that hypoxia increased calcium influx into human arterial endothelial cells and Remillard and Yuan [99] also implicated increased calcium influx. From these and other studies, Fantozzi et al. [103] conclude that such calcium influx plays a role in “stimulating pulmonary vascular cell proliferation and ultimately, in pulmonary vascular cell remodeling in patients with hypoxia-mediated pulmonary hypertension.” Bartsch et al. [104] showed that the calcium channel blocker nifedipine lowered high-altitude edema associated with high-altitude hypertension, suggesting an important role for elevated intracellular calcium in high-altitude PAH. Wang et al. [105] showed that cell proliferation and intracellular calcium levels were both increased by hypoxia, but that these responses were lowered by capsazepine, a specific antagonist of the TRPV1 receptor. They concluded that “TRPV1 may be a critical pathway or mediator in chronic hypoxia-induced proliferation of human pulmonary artery smooth muscle cells.” Hampl et al. [26] and Palmer et al. [106] showed that hypoxia induces iNOS in the pulmonary arteries. Loot and Fleming [107] showed that hypoxia-mediated vasoconstriction in the mouse was associated with increased calcium influx in mouse pulmonary arteries but that there was much lower calcium influx and much lower vasoconstriction when the arteries came from a transgenic mouse missing the TRPC6 receptor. All three of these studies implicate increased intracellular calcium in PAH and the latter two implicate two members of the TRP receptor family (TRPV1 and TRPC6), still another NO/ONOO<sup>-</sup> cycle element, in pulmonary hypertension. High-altitude and hypoxia lead to increased activity of the HIF transcription factor which lead, in turn to increased production of endothelin-1 in this condition [108]. As is discussed in the following section of this paper, endothelin-1 elevation leads to increases in most if not all of the NO/ONOO<sup>-</sup> cycle elements. It can be seen from the above two paragraphs that high-altitude/hypoxic pulmonary hypertension involves elevation of such NO/ONOO<sup>-</sup> cycle elements as elevated intracellular calcium, TRP receptor activity and iNOS induction; possible BH4 depletion must be viewed as hypothetical. However, other NO/ONOO<sup>-</sup> cycle elements may be implicated through the elevation of endothelin-1 in high altitude/hypoxic PAH.

Several *viral infections*, including HIV, HHV-8, and hepatitis B and C, are implicated in producing increased PAH incidence and prevalence [109–114]. It is estimated that HIV infection increases the prevalence of PAH approximately 2500-fold [110].

In the case of HIV, the viral transcriptional factor, Tat has been shown to lead to increased NF- $\kappa$ B activity, increased inflammatory cytokine production, increased 3-nitrotyrosine (a marker for peroxynitrite (ONOO<sup>-</sup>)), and increased oxidative stress [115]. Thus, all of these elements of the cycle can be produced through the expression of one viral protein. One of these specific cytokines that apparently has a causal role in HIV-associated PAH is IL-6 [116].

While it is clear, from the above, that the HIV-induced increase in PAH incidence and prevalence occurs independently of any antiretroviral drug and that such hypertension

can be reduced, in some cases by antiretroviral treatment, there is one antiretroviral drug that has a role in causing PAH. PAH has been shown to be caused by the anti-HIV protease drug ritonavir [117–119]. It apparently does this, at least in part, via increased oxidative stress, because multiple chain-breaking antioxidants greatly lower this effect of the drug [117–119]. Ritonavir has been shown to increase superoxide production [117, 120] in mitochondria [121], showing that both superoxide and mitochondrial dysfunction have roles here.

Bacterial infections can also have roles in initiation of cases of PAH. For example, pulmonary tuberculosis often leads to PAH [122–124] and not to, surprisingly, tuberculosis produces substantial elevation of much of the inflammatory cascade, including elevation of NF- $\kappa$ B inflammatory cytokines, iNOS induction, NO, and the marker of peroxynitrite (ONOO<sup>-</sup>), 3-nitrotyrosine [125].

The role of bacterial infection has been most studied in studies of the action of bacterial endotoxin causing PAH (see, e.g., [126–130]). Endotoxin exposure is known to produce major increases in NF- $\kappa$ B, inflammatory cytokines, iNOS induction leading to increased NO, ONOO<sup>-</sup>, and oxidative stress, and not surprisingly, these are all found in studies of endotoxin-induced lung injury including PAH [126–130]. Oxidative stress is specifically implicated in having a substantial causal role in initiation of PAH [128]. Furthermore, an important causal role may also be suggested for iNOS induction because of the action of a glucocorticoid in lowering PAH initiation [129]; it is well established that glucocorticoids can lower iNOS induction.

One of the most puzzling issues is the role of liver dysfunction in response to endotoxin exposure clearly shown by the study of Siore et al. [126]. The authors suggest that liver dysfunction may affect this response through such roles as bacterial or endotoxic clearance or roles in producing inflammatory cytokines and eicosanoids [126]. These may be a partial explanation, but there is another explanation for which there appears to be a better precedent. The most important function of the liver is thought to be detoxification of ammonia through the urea cycle and ammonia accumulation is known to be able to produce hepatic encephalopathy. Here ammonia action in the brain, acting via excessive activity of the NMDA receptors, produces the encephalopathy such that the encephalopathy can be greatly lowered by using NMDA antagonists [131–133]. It has been shown that high levels of inhaled ammonia can act to greatly increase lung dysfunction when present along with endotoxin exposure [134]. These considerations raise the question of whether liver dysfunction could cause PAH, in part through ammonia-caused stimulation of NMDA activity in the pulmonary arteries. It is known that there are NMDA receptors in the pulmonary arteries [135–137], making this interpretation plausible and of course this interpretation provides some support for the view that excessive NMDA activity, an important part of the NO/ONOO<sup>-</sup> cycle, may have an important role here.

The role of elevated homocysteine in PAH [138–140] also suggests but does not prove a role for the NMDA receptors in PAH, given the known role of homocysteine as an NMDA agonist. A study in pigs showed that a high methionine diet

which produces high serum homocysteine produced elevated PAH [141]. The lung dysfunction in this last study [141] was greatly lowered by an angiotensin-converting enzyme inhibitor, strongly suggesting a role for excessive superoxide in homocysteine-initiated PAH as well. This study showing that a simple high methionine diet may cause some cases of PAH suggests a possible initial cause for some cases of what are currently classified as idiopathic PAH and B vitamin/coenzyme deficiencies leading to homocysteine elevation should also be considered as a possible initial cause.

Poisoning by the herbicide paraquat has been shown to cause PAH, with the herbicide acting in an iNOS-dependent manner [142]. Paraquat toxicity also is known to involve mitochondrial dysfunction, excessive NMDA activity, oxidative stress, elevated inflammatory cytokines and elevated NF- $\kappa$ B activity [142–147], raising a possible role for all of these NO/ONOO<sup>-</sup> cycle elements in the generation of paraquat-dependent PAH.

Systemic autoimmune diseases, including systemic lupus erythematosus, systemic sclerosis and antiphospholipid syndrome are associated with greatly increased incidence of PAH [148–151]. Such autoimmune diseases are well known to increase the inflammatory parts of the cycle, including NF- $\kappa$ B inflammatory cytokine, and iNOS induction with these leading, in turn, to elevate ONOO<sup>-</sup> and oxidative stress [152]. They may act, therefore, via these mechanisms to turn on the NO/ONOO<sup>-</sup> cycle.

Inherited cases of PAH occur in PAH families and roughly 3/4 of these are caused by mutations in the BMPR2 gene [153]. Such mutations in this gene, are scattered through much of the gene, such that functions associated with specific parts of the protein encoded by the gene can be ruled out [154]. Because of this, before the recent study of Lane et al. [154], there was no common consequence of the diverse mutations implicated in causing PAH. Lane et al. [154] studied a series of toxic dominant gain of function mutants causing PAH and showed that these all generated increased superoxide-dependent oxidative stress and also mitochondrial dysfunction. They argue that the superoxide-dependent oxidative stress is probably causally involved in PAH and that mitochondrial dysfunction is likely to be causal as well. Thus, three elements of the NO/ONOO<sup>-</sup> cycle appear to be implicated in this mechanism of disease initiation, namely, increased superoxide, oxidative stress, and mitochondrial dysfunction. It should be noted that genetic initiation involves a very long-term stressor, not a short-term one.

Serotonin (5-HT) and agents that raise serotonin levels and are thought to act via serotonin in case PAH initiation. These agents include fenfluramine, L-tryptophan, cocaine, monochromaline, and amphetamines, which are all thought to act via elevated serotonin to initiate cases of PAH [155–161]. Oxidative stress responses are involved here [148, 162] as are increased levels of inflammatory cytokines [163]. Serotonin is thought to act, at least in part, by raising levels of a regulatory peptide known as RhoA [155, 156, 161]. RhoA is also thought to have roles in several other types of PAH cases including cases involving bleomycin, hypoxia, and endotoxin exposure

[162, 164–167] and may, therefore, play a general causal role in PAH. The only way that RhoA can have a causal role in PAH generally, if PAH is a NO/ONOO<sup>-</sup> cycle disease, is if RhoA acts as part of the cycle.

It is important, therefore, to determine whether RhoA levels may be elevated in response to NO/ONOO<sup>-</sup> cycle elements and also whether it, in turn, may elevate NO/ONOO<sup>-</sup> cycle elements. Both of these are predicted if RhoA is acting as part of the NO/ONOO<sup>-</sup> cycle.

RhoA activity is increased by NF- $\kappa$ B [168, 169] and also by the inflammatory cytokines TNF- $\alpha$  [169–172] and IL-13 [169, 170]. Both of these cytokines act by raising NF-kappaB when they raise RhoA activity. [168–170]. The inflammatory marker C-reactive protein increased RhoA/Rho-kinase signaling [173]. Jin et al. [174] showed that various free radicals and reactive oxygen species increased RhoA/Rho-kinase signaling. Ryoo et al. [175] showed that oxidative stress, acting through oxidized LDL, stimulated RhoA signaling.

There are a number of studies reporting that RhoA/Rho kinase have roles in generating NO/ONOO<sup>-</sup> cycle elements, with some of them being done in the context of its role in the vascular epithelia but others done in other pathophysiological contexts. For example, Chandra et al. [176] showed that RhoA/Rho kinase produced increases in ONOO<sup>-</sup>, superoxide and consequent hydrogen peroxide, and oxidative stress. Resta et al. [177] and also Broughton et al. [178] reported RhoA-dependent apparent increases intracellular calcium levels and also superoxide levels. A Rho kinase inhibitor was shown to decrease both superoxide levels and BH4 depletion and consequent eNOS uncoupling, strongly suggesting that RhoA/Rho kinase raise both superoxide and deplete BH4 [179]. RhoA/Rho kinase increase activity of the inflammatory cytokine/chemokine IL-8 [168].

It can be seen, from the above, that RhoA and RhoA-dependent signaling is both stimulated by three elements of the NO/ONOO<sup>-</sup> cycle (NF- $\kappa$ B, cytokines, and oxidants/oxidative stress) and that they in turn stimulate multiple elements of the NO/ONOO<sup>-</sup> cycle, including superoxide, ONOO<sup>-</sup>, oxidative stress, BH4 depletion, and elevated intracellular calcium, providing support for the view that RhoA functions in these tissues as part of the NO/ONOO<sup>-</sup> cycle. A similar view was proposed by Yao et al. [180], who in Figure 1 of that paper showed RhoA and the Rho kinase as part of a vicious cycle, with RhoA being stimulated by reactive oxygen species and the cytokine TNF-alpha and RhoA acting in turn, to raise various inflammatory cytokines and other markers, endothelial dysfunction (which is known to involve BH4 depletion) and various oxidants. Elsewhere in that paper [180], they have elevated NF- $\kappa$ B activity as part of their cycle.

The drug bleomycin has been shown to initiate some cases of PAH [162, 181]. It is known to increase several mechanisms involved in the NO/ONOO<sup>-</sup> cycle including stimulating poly (ADP-ribose) polymerase (PARP), oxidative stress, superoxide generation, inflammatory cytokines, oxidative stress, partial uncoupling of the NOSs (which is presumably caused by BH4 depletion), mitochondrial dysfunction, and NF- $\kappa$ B elevation [181–185]. Superoxide is

specifically implicated in having a causal role in bleomycin-initiated PAH because overexpression of superoxide dismutase in a mouse model lessens subsequent pulmonary hypertension, fibrosis, and vascular remodeling following bleomycin treatment [185]. The most directly affected of these is the PARP mechanism because it is greatly stimulated by the single- and double-strand breaks in DNA that are produced by bleomycin. In addition, apoptosis, which is sometimes involved in NO/ONOO<sup>-</sup> cycle diseases, is also triggered by bleomycin [183]. It can be seen from this, that most of the NO/ONOO<sup>-</sup> cycle is triggered by bleomycin, such that this alone strongly suggests a NO/ONOO<sup>-</sup> cycle mechanism for PAH.

An animal model of PAH is caused by a mutation in a gene that produces a deficiency in the production of vasoactive intestinal peptide (VIP) [186, 187]. VIP was also shown to aid in therapy of PAH [188] and a VIP deficiency was shown to produce an inflammatory response [187]. VIP has been shown to stimulate the production of BH4, acting through the synthesis of the enzyme GTP cyclohydrolase I, the rate limiting step in the *de novo* production of BH4 [189]. VIP has also been shown to lower NF- $\kappa$ B activity, inflammatory cytokines and oxidative stress [190, 191]. It follows that a deficiency in VIP will be expected to produce a deficiency in BH4, raise NF- $\kappa$ B activity, inflammatory cytokine levels, and oxidative stress, with all of these being important NO/ONOO<sup>-</sup> cycle elements.

The studies discussed in the preceding paragraph suggest a causal role for a BH4 deficiency in PAH, but do not prove such a role. However, genetic studies on a BH4-deficient mouse mutant (hph-1), clearly show such a causal role [102, 192, 193]. BH4 deficiency is the well-established cause of nitric oxide synthase uncoupling, leading to increased superoxide production. eNOS uncoupling, a consequence of BH4 depletion, is also found in PAH [194]. BH4 depletion causes lowered eNOS expression (reviewed in [193]), a common correlate found in PAH.

The elements of the cycle implicated in the action various initiators of PAH are summarized in Table 1. It can be seen from Table 1 that each of the elements of the cycle is implicated in action of at least one PAH initiator with most being implicated in multiple initiators. Even PARP, which is often not listed as a NO/ONOO<sup>-</sup> cycle element but has a major role in one of the two cascades of events leading to mitochondrial dysfunction, is elevated in response to one of the initiators, bleomycin.

While each of the cycle elements is implicated in the action of initiators of PAH, that does not necessarily mean that they all have important causal roles in PAH. It is possible that some may be epiphenomena, occurring but not playing any causal role. It is important, therefore, to look at which elements have causal roles in the initiation process, based either on genetic studies or on the use of specific inhibitors, or both. The studies cited above in this section provide evidence for causal roles in PAH initiation for BH4 depletion, superoxide elevation, oxidative stress, TRPV1 and TRPC6 activity, and the cytokine IL-6.

TABLE 1: NO/ONOO<sup>-</sup> cycle elements implicated in PAH initiation.

Initiator	NO/ONOO <sup>-</sup> cycle element.
High altitude/hypoxia	Increased intracellular calcium, TRP receptor activity elevation, iNOS induction, (BH4 depletion?)
Viral infection	NF-κB elevation, elevated inflamm. cytokines, iNOS induction, NO elevation, ONOO <sup>-</sup> elevation, oxidative stress
Ritonavir	Oxidative stress, superoxide elevation, mitochondrial dysfunction
Tuberculosis	NF-κB elevation, elevated inflamm. cytokines, iNOS induction, NO elevation, ONOO <sup>-</sup> elevation
Endotoxin	NF-κB elevation, elevated inflamm. cytokines, iNOS induction, NO elevation, ONOO <sup>-</sup> elevation, oxidative stress, (NMDA elevation?)
Homocysteine	NMDA elevation, superoxide elevation
Paraquat	Mitochondrial dysfunction, elevated NMDA activity, oxidative stress, elevated inflamm. cytokines, NF-κB elevation
Systemic autoimmune disease	Oxidative stress, inflamm. cytokine elevation; ONOO <sup>-</sup> elevation, superoxide elevation, inflamm. cytokine elevation, BH4 depletion, elevated intracellular calcium, NF-κB elevation
BPMR2 mutation	Superoxide, oxidative stress, mitochondrial dysfunction
Serotonin	Oxidative stress, inflamm. cytokine elevation, ONOO <sup>-</sup> elevation, superoxide elevation, inflamm. cytokine elevation, NF-κB elevation
Bleomycin	PARP elevation, oxidative stress, superoxide elevation, inflamm. cytokine elevation, BH4 depletion, mitochondrial dysfunction, NF-κB elevation
VIP deficiency	BH4 deficiency, inflammatory cytokine elevation
BH4 deficiency	BH4 deficiency, superoxide elevation

## 6. Endothelin-1 as an Important Causal Factor in PAH

Endothelin-1 (ET-1) is a potent vasoconstrictor 21-amino acid residue protein, generated by proteolysis of a larger precursor protein [103A]. It has an important role in regulating vascular tone [195, 196], and its levels have been shown to be substantially elevated in PAH [197]. Furthermore, an ET-1 receptor antagonist is thought to be an effective treatment for PAH [198]. These observations and many others that have confirmed them argue strongly that ET-1 has an important causal role in PAH. And this, in turn, provides both an important challenge and important test of the NO/ONOO<sup>-</sup> cycle mechanism for PAH.

The only way that the NO/ONOO<sup>-</sup> cycle can be causal for PAH if ET-1 elevation is also causal is for ET-1 elevation to be part of the cycle in PAH. That is, the NO/ONOO<sup>-</sup> cycle, if it applies to PAH, predicts that ET-1 levels must be elevated in response to one or more cycle elements and must act, in turn, to raise various cycle elements. These are both strong predictions of the NO/ONOO<sup>-</sup> cycle mechanism, if it applies to PAH, strong predictions that are not likely to be made based on any other unrelated hypothesis. Are they both correct? The following studies involve the ET-1 role in PAH but also in other tissues.

ET-1 has been shown to be induced by inflammatory cytokines [199–201]. It has also been shown to be induced by oxidants with such induction also lowered by antioxidants [202–204]. The activity of ET-1 has been shown to be increased by both NF-κB action and also the action of another inflammatory cytokine activated pathway [205–207]. Thus, the inflammatory part of the NO/ONOO<sup>-</sup> cycle has a powerful role in increasing ET-1 activity. Furthermore

BH4 was shown to substantially lower ET-1 levels [208], so that BH4 depletion, another part of the NO/ONOO<sup>-</sup> cycle has a role in raising ET-1 levels. ET-1 levels are also reported to be increased by TRPV1 [209, 210], another part of the cycle. It follows from these observations that elements of the NO/ONOO<sup>-</sup> cycle (at least five, in this case) have substantial roles in raising ET-1 levels, as predicted if PAH is a NO/ONOO<sup>-</sup> cycle disease and that ET-1 elevation also has a causal role in PAH.

Furthermore, ET-1 action has been shown to elevate, in turn, essentially the entire NO/ONOO<sup>-</sup> cycle. It increases oxidative stress [211–217], superoxide [211, 213–216], the ONOO<sup>-</sup> marker 3-nitrotyrosine [211, 212], NF-κB activity [211, 217], and NO [211, 213, 218], lowers levels of BH4 [213–215, 217, 219], stimulates iNOS induction [211, 217], increases TRP receptor function [220, 221], increases levels of intracellular calcium [222–224], increases NMDA activity [225, 226], produces mitochondrial dysfunction [227, 228], and increases inflammatory cytokine levels [206, 207, 217]. All of these provide evidence for elevation of each of the NO/ONOO<sup>-</sup> cycle elements.

However, there is one fact that requires some explanation. Even though there are studies showing that ET-1 increases iNOS induction [211, 217], there are also studies showing that it can decrease iNOS induction [219, 229]. These latter studies are in situations where substantial iNOS was already induced before ET-1 exposure and where lowered iNOS induction was accompanied by BH4 depletion [219, 229]. It has been shown that high level iNOS induction requires substantial pools of BH4 [230]. It is the author's view, therefore, that this lowered iNOS induction is probably caused by the BH4 depletion produced by ET-1 and therefore that these observations do not conflict with the conclusion that ET-1 acts by elevating

the NO/ONOO<sup>-</sup> cycle. ET-1 lowers induction of the enzyme GTP cyclohydrolase I, the rate limiting step in the production of BH4 [216], providing further evidence for a BH4 role here.

In conclusion, then, there is substantial evidence that elevated ET-1 can be induced by five elements of the NO/ONOO<sup>-</sup> cycle and that it can act, in turn, to elevate all of the NO/ONOO<sup>-</sup> cycle elements. It follows that ET-1 acts, at least in part, by elevating the NO/ONOO<sup>-</sup> cycle in PAH.

## 7. Elevated NO/ONOO<sup>-</sup> Cycle Elements in the Chronic Phase of PAH and Therapy via Agents that Lower NO/ONOO<sup>-</sup> Cycle Elements

The *second principle* of the NO/ONOO<sup>-</sup> cycle is that the elements of the cycle should be elevated in the chronic phase of the disease. And the *fifth principle* is that NO/ONOO<sup>-</sup> cycle diseases should respond to therapies that lower NO/ONOO<sup>-</sup> cycle elements and should, therefore, be treated in this way. The studies supporting both of these principles in PAH often deal with both of them, so both of these principles will be treated together here. We have already discussed substantial evidence for this in PAH in the context of the action of the various stressors that initiate PAH—evidence discussed in the previous two sections of this paper. Many of the studies cited in those two sections are not only relevant to the issue of disease initiation but also are relevant to the issue of the chronic phase of the disease. This section considers still additional studies that relate to these same issues, are elements of the cycle elevated in the chronic phase of PAH? Are agents that lower NO/ONOO<sup>-</sup> cycle elements helpful in therapy. Much of this discussion focuses on animal model studies.

Bowers et al. [28] was a histological study of human lung tissue in PAH, which showed that several markers of oxidative stress were elevated, that 3-nitrotyrosine, a marker of ONOO<sup>-</sup> was elevated and that inflammatory markers were elevated as well. They suggest that [28] chronic prostacyclin infusion, an anti-inflammatory treatment is helpful, suggesting that agents lowering the inflammatory part of the cycle may be useful in therapy.

Lakshminrusimha et al. [231] found that isoprostane, a marker of oxidative stress, and 3-nitrotyrosine, a marker of ONOO<sup>-</sup>, were both elevated in PAH and that addition of intratracheal superoxide dismutase (SOD) an enzyme that degrades superoxide, produced symptomatic improvement and lowered both of these markers. Interestingly, they also showed that inhaled NO produced symptomatic improvement but also raised ONOO<sup>-</sup>.

Herget et al. [232] reported increased NO production and elevated 3-nitrotyrosine in hypoxic PAH and also showed that antioxidants were helpful in prevention of PAH. This study, implicates NO, ONOO<sup>-</sup>, and oxidative stress and suggests that antioxidants may be helpful in therapy.

Weissmann et al. [233] reviewed evidence that both superoxide and its product, hydrogen peroxide, are elevated in PAH and argue that both mitochondrial and extramitochondrial mechanisms, including NADPH oxidase, are

involved in superoxide generation. They also argue for a role of oxidant damage in causing the tissue remodeling in PAH.

Van Rheen et al. [185] studied normal mice and those expressing much higher levels of extracellular superoxide dismutase (SOD) in PAH initiated by bleomycin. The vascular remodeling and PAH were greatly lowered by SOD and survival was greatly improved. These studies provide strong support for the view that superoxide has an important role in tissue remodeling and PAH and that an agent that acts to lower superoxide is, therefore very helpful in prevention.

Dorfmueller et al. [234] showed that a marker of oxidative stress, malondialdehyde, 3-nitrotyrosine (ONOO<sup>-</sup> marker), and an inflammatory cytokine were all elevated in the tissue remodeling in PAH. There were also changes in expression of antioxidant enzymes, providing further support for a role of oxidative stress.

Rashid et al. [235] showed that a superoxide dismutase mimic greatly lowered the development of PAH, suggesting a causal role for superoxide and showing that an agent that lowers superoxide was very useful in prevention.

Lu et al. [236] showed that the drug, rosiglitazone, a PPAR $\gamma$  agonist, has favorable properties on human pulmonary artery smooth muscle, acting to lower two responses that are both elevated in response to hypoxia: increased NF- $\kappa$ B activity and increased expression of one of the NADPH oxidase genes, Nox4. Rosiglitazone also acts to lower PAH responses. Because NADPH oxidase produced superoxide, this also implicates superoxide in PAH. These studies show that an agent that lowers both superoxide production and NF- $\kappa$ B activity is helpful in therapy.

*Oxidative stress* and reactive oxygen species were discussed as causal factors in generating the symptoms and signs of PAH in the introduction to this paper [17, 22].

**7.1. Peroxynitrite (ONOO<sup>-</sup>).** There are many papers, showing the 3-nitrotyrosine, a marker for ONOO<sup>-</sup> elevation, is raised in PAH, including four that were discussed above in this section [28, 185, 231, 232]. In another study, Oishi et al. [237], showed that both superoxide and ONOO<sup>-</sup> were elevated following rebound from NO inhalation. So there should be no question that ONOO<sup>-</sup> is elevated in PAH. What is the main focus, here, therefore, is the study of *acausal role of ONOO<sup>-</sup>* in PAH.

Agbani et al. [238] showed that ONOO<sup>-</sup> stimulated the growth of pulmonary artery smooth muscle cells, a response involved in the remodeling response in PAH; the growth response was blocked by ebselen, an ONOO<sup>-</sup> scavenger, providing further evidence for a causal role for ONOO<sup>-</sup>.

Aggarwal et al. [239] showed that addition of an NO donor to pulmonary artery endothelial and smooth muscle cells increased ONOO<sup>-</sup> and increased nitration of protein kinase G. This response was shown to be blocked by either superoxide dismutase (SOD) or by an SOD mimic drug. This study followed an earlier one showing that nitration of the G kinase lowered its responsiveness to cGMP stimulation and therefore lowered its ability to produce NO-stimulated vasodilation and such attenuated vasodilation was also demonstrated in this study [239]. One part of the study showed that inhaled NO produced elevation of ONOO<sup>-</sup>,

increased G kinase nitration, and decreased G kinase activity, providing a mechanism for lowered responsiveness to inhaled NO with time. Interestingly, in one part of the study [239], L-arginine was found to lower both ONOO<sup>-</sup> following simultaneous NO inhalation and also helped to maintain G kinase activity; this is important because L-arginine increases NO synthesis while lowering superoxide production from uncoupled nitric oxide synthases; the arginine finding argues, therefore, that superoxide generated on the uncoupled nitric oxide synthase enzyme may have a key role here. The causal role of ONOO<sup>-</sup> here is demonstrated by its known role in protein nitration, the demonstrated causation of lowered G kinase activity by a compound that breaks down to produce ONOO<sup>-</sup> and also the demonstrated causal role in this study of both of the ONOO<sup>-</sup> precursors, NO, and superoxide. This is a clear study demonstrating that an important change in the vasculature in PAH, namely, lowered responsiveness to NO mediated vasodilation, is caused by ONOO<sup>-</sup> mediated nitration of a particular protein, the G kinase. It also shows the efficacy of agents lowering superoxide.

In another study, Agbani et al. [240] demonstrated that ONOO<sup>-</sup> stimulates pulmonary endothelial cell and smooth muscle proliferation by a regulatory pathway involving both ERK and PKC protein kinases as well as two growth factor receptors. The causal role of ONOO<sup>-</sup> was demonstrated here, both by adding pure peroxyxynitrite (ONOO<sup>-</sup>) and by using the ONOO<sup>-</sup> scavenger ebselen to block these responses [240]. Belik et al. [241] asked whether hypoxia-caused PAH and vascular remodeling were caused by ONOO<sup>-</sup> elevation by using a ONOO<sup>-</sup> decomposition catalyst, FeTPPS. They showed that FeTPPS blocked some but not other changes [241], showing that those that were blocked were probably produced by ONOO<sup>-</sup>. Masood et al. [242] used this same FeTPPS decomposition catalyst to determine whether ONOO<sup>-</sup> was responsible for various changes in the pulmonary vasculature produced by 60% oxygen exposure in neonatal rats, leading to PAH. They concluded that some but not other changes were caused by ONOO<sup>-</sup>. It may be inferred from the above studies that many of the changes seen with PAH in the pulmonary vasculature are caused by ONOO<sup>-</sup>, the most central element in the NO/ONOO<sup>-</sup> cycle but that there are others that are not caused by ONOO<sup>-</sup> but perhaps may involve other cycle elements.

**7.2. NF- $\kappa$ B.** One study discussed earlier in this section, Lu et al. [236] implicated elevated NF- $\kappa$ B activity in PAH as did several studies cited in the previous two sections of the paper. There are several additional studies that implicate elevated NF- $\kappa$ B activity in PAH. In one of these, Liu et al. [243] studied increased NF- $\kappa$ B expression that developed along with PAH. The statin drug simvastatin was shown to have a favorable effect, apparently in part at least, by suppressing the increase in NF- $\kappa$ B expression. A study by Li et al. [244] was not a study on PAH but was a study of the effect of an inflammatory protein found in PAH, C-reactive protein on the expression of NF- $\kappa$ B and also the inflammatory cytokine IL-6 in pulmonary artery smooth muscle cells. They showed that both NF- $\kappa$ B expression and IL-6 expression were raised by C-reactive protein and that these raises were suppressed

by another statin drug, atorvastatin. Both of these studies provide some support for the view that increased NF- $\kappa$ B expression and in the latter study, the cytokine IL-6, may have roles in PAH and that suppressing these roles with statin drugs may be useful therapeutically.

Another study suggesting a causal role of NF- $\kappa$ B elevation is that of Kimura et al. [245], which showed that a nanoparticle-mediated delivery of an NF- $\kappa$ B decoy (i.e., a nucleic acid-like sequence to which NF- $\kappa$ B binds) to the lungs, greatly attenuated inflammation and smooth muscle proliferation in a monocrotaline PAH model. They also reported NF- $\kappa$ B activity elevation in the lungs of patients with PAH.

Sawada et al. [246], studying a monocrotaline rat model of PAH, found prolonged elevation of NF- $\kappa$ B activity. They used the NF- $\kappa$ B inhibitor pyrrolidine dithiocarbamate and showed that it lowered NF- $\kappa$ B activity and ameliorated the PAH development. Their results both confirm a role of NF- $\kappa$ B elevation in PAH and suggest that agents lowering NF- $\kappa$ B activity should be useful in therapy.

Huang et al. [247] did a similar study, also in a monocrotaline rat PAH model but got very different results. They found only a brief elevation of NF- $\kappa$ B activity following monocrotaline treatment and did not confirm the lowering of NF- $\kappa$ B activity by pyrrolidine dithiocarbamate. They state that the “reason for the discrepancy between our results and those of Sawada et al. is not clear.”

Two additional studies showed that NF- $\kappa$ B activity can produce vascular smooth muscle proliferation [248, 249]. In one of these studies [248], antisense polynucleotides blocked expression of one of the protein subunits of NF- $\kappa$ B and blocked vascular smooth muscle proliferation. In the other study [249], a hyperactive mutant gene for the inhibitor of NF- $\kappa$ B IkappaB $\alpha$ , greatly lowered NF- $\kappa$ B activity and also blocked vascular smooth muscle proliferation. These studies show that vascular smooth muscle proliferation can be caused by NF- $\kappa$ B activity elevation and also lowered by agents that lower NF- $\kappa$ B activity.

In summary, there is substantial evidence that elevated NF- $\kappa$ B activity has a role in PAH and that it has a causal role in the development of PAH, being specifically implicated in smooth muscle proliferation and therefore in tissue remodeling.

**7.3. Inflammatory Cytokines.** There have been numerous studies reporting elevated levels of *inflammatory cytokines* in PAH, including two discussed above in this section [234, 243]. A number of such studies reported elevation of multiple inflammatory cytokines/chemokines in PAH. For example, Soon et al. [250] reported that IL-1 $\beta$ , -2, -4, -6, -8, -10, and -12p70, as well as TNF- $\alpha$  were elevated in PAH patients. Elevated levels of four of these were associated with lowered survival, with IL-6 showing the highest such association. Li et al. [251] found that 11 inflammatory proteins were elevated in adventitial fibroblasts from calves suffering from hypoxic PAH, most of these proteins different from the ones studied by Soon et al. [250], but including IL-1 $\beta$  and IL-6. Yu et

al. [252] reported elevated levels of IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 in PAH patients undergoing hemodialysis. Sin and Man [253] also provided evidence for elevation of IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 in PAH patients. Hamal et al. [254] showed that PAH induced by injection of microparticulate cellulose into susceptible chickens was accompanied by elevated expression of several inflammatory cytokines including IL-1 $\beta$ , IL-4, IL-6, IL-8, and IFN $\gamma$ . An IL-1 receptor antagonist was shown to lower PAH responses, suggesting a causal role for IL-1 $\beta$  elevation in PAH [255]. Wanderer provided a strong rationale for using IL-1 $\beta$  antagonists or receptor blockers in PAH treatment [256].

A number of studies have focused, to some extent, specifically on a role for IL-6 in PAH. Kalambokis et al. [257] reported high serum levels of IL-6 in cirrhosis-associated PAH. Chaouat et al. [258] found that COPD patients with PAH had higher levels of IL-6 than did COPD patients without PAH. They also showed that individuals carrying a genetic polymorphism in the IL-6 gene associated with higher gene expression had significantly higher prevalence of PAH, strongly suggesting a causal role for IL-6. Finally they showed [258] that pulmonary artery smooth muscle cells exposed to hypoxic conditions had twice the IL-6 mRNA as those not exposed to hypoxic conditions. They concluded that “inflammation, most likely involving IL-6, may contribute substantially to PH complicating COPD.” Furuya et al. [259] reviewed evidence implicating IL-6 in promoting smooth muscle and epithelial proliferation in PAH. They showed that a patient with severe refractory PAH responded dramatically to treatment with a humanized monoclonal antibody to the IL-6 receptor and suggest that IL-6 blockade may be a promising adjunct treatment for PAH. Steiner et al. [260] compared transgenic mice overexpressing IL-6 with normal mice. They found that such transgenic mice differed from normals under both hypoxic and normoxic conditions, leading to increased occurrence of a number of changes characteristic of PAH. They suggest that “IL-6 promotes the development and progression of pulmonary vascular remodeling and PAH through proliferative and antiapoptotic mechanisms.”

It may be seen from the above two paragraphs, that there are wide ranging proinflammatory changes associated with PAH and that IL-6 elevation may play a particularly important role in PAH development. One study [259] suggests that IL-6 action may be an important therapeutic target in PAH and two other studies [255, 256] suggest that IL-1 $\beta$  action may be as well.

**7.4. iNOS Induction.** iNOS induction has been reported in two additional studies to be implicated in PAH [261, 262]. In one of these [262], a specific inhibitor of iNOS was shown to greatly lower PAH development, arguing for a causal role of iNOS induction in PAH. An iNOS knockout mouse was less sensitive to exacerbation of PAH, including inflammatory responses, as discussed above [263].

**7.5. Mitochondrial Dysfunction.** Quite a number of studies have reported substantial mitochondrial dysfunction in PAH

[264–269]. The genetic studies in [266–268] strongly suggest that such mitochondrial dysfunction can have a strong, causal role in PAH and one study used mitochondrial inhibitors which showed a complex causal role in PAH [269]. Four of these studies [264, 266, 268, 269] all suggest mechanisms by which mitochondrial dysfunction may contribute to the symptoms and signs of PAH. However, none of these provide any evidence that agents that improve mitochondrial function can prevent or produce improvements in PAH. This last issue of possible therapy via improved mitochondrial seems to be a neglected one in PAH. The only such studies that the author is familiar with are two studies showing that inhibitors of poly (ADP-ribose) polymerase (or synthetase) (PARS or PARP) provide substantial protection from PAH [270, 271]. This PARP activity is known to initiate one of the two cascades of events, leading to mitochondrial dysfunction, proposed to be part of the NO/ONOO $^-$  cycle, and therefore lowering its activity will be predicted to have a major effect in lowering mitochondrial dysfunction, as has been shown in a number of chronic inflammatory diseases.

**7.6. TRP Receptors and Also Intracellular Calcium.** The study of Yu et al. [272] showed that a single nucleotide polymorphism in the TRPC6 gene, introducing an NF- $\kappa$ B site in the enhancer region of the gene, increased susceptibility to PAH. This study implicates both a TRP receptor and consequent increased intracellular calcium in PAH. Two studies, in addition to the ones cited above, also suggest roles of the TRP group of receptors and increased intracellular calcium in PAH [273, 274]. Capsaicin is a TRPV1 agonist which over the longer term, produces very substantial down-regulation of the TRPV1 and several other TRP receptors. It is interesting, therefore that several studies showed that capsaicin pretreatment over a period of days produces greatly decreased PAH induction [275–277].

There are a variety of additional studies showing that elevated intracellular calcium levels have causal roles in PAH [273–282], including studies using calcium channel blockers [279–282] to obtain clinical improvement. It follows that agents lowering intracellular calcium may also be useful in therapy. A role for intracellular calcium is further supported by a study showing that inhibition of calcium-dependent protease, calpain blocked the development of several features of PAH [283].

**7.7. NO Elevation?** The NO/ONOO $^-$  cycle mechanism predicts that NO synthesis will be increased through iNOS induction and by calcium-stimulation of eNOS and nNOS activity but that it will also be decreased through BH4 depletion and consequent partial uncoupling of all three NOSs [8]. Furthermore, as discussed above, BH4 depletion can lead to lowered eNOS and iNOS expression. Consequently, it is unclear whether the cycle mechanism should predict whether an increase or decrease in NO should be expected. However, most proposed NO/ONOO $^-$  cycle diseases have published studies showing apparent increases in NO synthesis.

It is important to raise the question as to whether NO should be considered an element of the cycle or not. Clearly,

the answer is yes, if one considers that NO is essential for the production of peroxynitrite ( $\text{ONOO}^-$ ), but the answer is less clear as to whether its synthesis must be elevated in NO/ $\text{ONOO}^-$  cycle diseases. These considerations should be kept in mind in considering the data on NO in PAH.

Most studies of NO in PAH patients have measured exhaled NO levels from the lungs in order to assess possible changes in NO synthesis in PAH [284–290]. NO is relatively stable in the gas phase, allowing for such measurements, whereas the instability of NO in biological fluids means that estimates of NO synthesis from blood measurements are usually done via nitrate/nitrite products derived from NO. However the instability of NO in biological fluids means that anything that lowers the movement of NO from the pulmonary arteries into the gas phase, such as fibrosis, arterial remodeling, or lowered ciliary activity may be expected to lower measured exhaled NO. Furthermore, the reaction of NO with superoxide to form  $\text{ONOO}^-$  (both superoxide and  $\text{ONOO}^-$  are clearly both elevated in PAH), will also lower any measurement of exhaled NO.

The majority of studies on exhaled NO in PAH patients report lowered levels when compared with controls [284–287, 289], but two such studies report apparent elevated levels, with one reporting a nonsignificant trend [288] and the other significant elevation [290]. Consequently, there appears to be substantial variation in these studies. Two of these studies compared exhaled NO levels with nitrate/nitrite levels in the blood in the same individuals. Both found that although exhaled NO levels were lower in PAH patients, blood levels of nitrite/nitrate were elevated [284, 289]. The interpretations of these results by the authors of these two studies were different from one another, but clearly one interpretation is the one suggested in the previous paragraph, that NO synthesis may be elevated but that movement of NO from the pulmonary arteries to the gas phase may be substantially slowed in PAH. If this interpretation is correct, it raises questions about whether any of the studies of lowered exhaled NO in PAH should be interpreted as good measurements of NO synthesis. In addition, three studies reported that therapies that produced symptomatic improvement in PAH patients, lowered exhaled NO levels [285, 291, 292], suggesting a correlative relationship between lowered NO and improved symptoms.

The observations discussed in the previous two paragraphs show that NO is not likely to be consistently low in PAH and that correlative data suggest that therapies lowering NO can produce therapeutic improvement, although none of these therapies were designed to lower NO. Before leaving this issue, it is important to discuss an additional type of observation on PAH. It has often been reported that lowered eNOS expression is found in the pulmonary arteries and associated cells in PAH, and such observations have often been used to suggest that low NO helps cause PAH. However, it should be noted that BH4 depletion, a part of the cycle that has been confirmed in PAH, causes lowered expression of eNOS (reviewed in [193]). It is possible, therefore, that such lowered eNOS expression is a protective mechanism, aimed at avoiding excessive superoxide production, rather than a causal mechanism in PAH; if this view is correct,

then it follows that this protective mechanism is insufficient to avoid PAH under conditions where the disease is induced.

In conclusion, then, none of the studies on NO in PAH can be argued to conflict with the NO/ $\text{ONOO}^-$  cycle mechanism, although like many other types of data, there are multiple interpretations of these data that can be suggested.

One of the questions that must be raised in the context of a possible NO/ $\text{ONOO}^-$  cycle mechanism for PAH is what role NO has in the process? Clearly inhaled NO can produce an initial lowering of hypertension, something that might be expected, based on the role of NO as a vasodilator and also possibly the action of NO in lowering two NO/ $\text{ONOO}^-$  cycle elements, NF- $\kappa$ B activity and NMDA activity. However, the most crucial NO role in the cycle is acting as a precursor for  $\text{ONOO}^-$  and consequently a pathophysiological role for NO is an essential part of the cycle. Such a pathophysiological role for NO in PAH is most clearly seen in the rebound that occurs after NO inhalation, when NO is no longer producing favorable responses and therefore is no longer inhaled. The rebound response to previous NO inhalation clearly shows that NO can produce a chronic exacerbation of the disease and clearly establishes such a pathophysiological role. However, a question should be raised is whether the properties of that chronic exacerbation are consistent with that predicted by the NO/ $\text{ONOO}^-$  cycle. There are a number of studies that suggest that they are. For example, Weinberger et al. [293] showed that previous NO exposure led not only to elevated  $\text{ONOO}^-$ , as predicted by the NO precursor role in  $\text{ONOO}^-$  formation, but also increased superoxide production and iNOS activity, consequences that must be indirect but are predicted by the cycle. Wedgwood et al. [212] showed a role for both increased  $\text{ONOO}^-$  and superoxide in the rebound response following NO inhalation. Shanley et al. [263] showed inflammatory responses following NO inhalation, responses that were greatly lowered in an iNOS knockout mouse, showing that such inflammatory responses were not only produced by such NO inhalation, but were lowered by blocking an important part of the NO/ $\text{ONOO}^-$  cycle, namely iNOS induction. The Lakshminrusimha et al. study [231] showed that inhaled NO produced increases in  $\text{ONOO}^-$ . The Aggarwal et al. study [239] showed that inhaled NO not only raised  $\text{ONOO}^-$ , but also was  $\text{ONOO}^-$  dependent nitration of the G kinase enzyme, leading to lowered vasodilation, an important part of the rebound phenomenon. Oishi et al. [237] found that both  $\text{ONOO}^-$  and superoxide were elevated following NO inhalation and established a causal role of superoxide in the rebound response by using polyethylene glycol-conjugated SOD to inhibit the rebound response.

All of these findings are consistent with upregulation of the NO/ $\text{ONOO}^-$  cycle as a consequence of inhaled NO, with NO acting as a  $\text{ONOO}^-$  precursor. The knockout iNOS mouse study suggests that lowering NO can be helpful and can lower the inflammatory parts of the cycle.

This does not mean that there are no situations where NO can be helpful, certainly its role in vasodilation and its potential role in lowering both NMDA receptor activity and NF- $\kappa$ B activity can also be helpful, but in a NO/ $\text{ONOO}^-$

cycle situation, its role as a precursor of  $\text{ONOO}^-$  must be considered to be paramount.

**7.8. BH4 Therapy.** Several studies implicating BH4 depletion in PAH were discussed in the previous two sections of this paper. Three studies suggest that agents that raise BH4 levels may be helpful in therapy. One was a study on the safety of sapropterin (the drug name for BH4) in PAH patients [294].

In another paper, Teng et al. [295] studied the pulmonary artery endothelial tissues from fetal lambs with pulmonary hypertension comparing these is normal lambs. In this study Teng et al. [295] showed that the BH4 levels in the PAH lambs were very low compared with normals, leading to eNOS uncoupling, lowered NO and raised superoxide. Interestingly, the lowered BH4 levels were not only produced by  $\text{ONOO}^-$  oxidation of BH4, but also by lowered GTP cyclohydrolase I, the rate limiting enzyme in BH4 synthesis. Addition of sepiapterin, a BH4 precursor to these PAH arterial tissues, raised BH4, raised NO synthesis and lowered superoxide. It also led to increased probable stability of the eNOS enzyme by increased association with Hsp90 protein. Most importantly it help restore angiogenesis which is deficient in PAH. The authors suggest that raising BH4 levels is important in PAH therapy.

A third study showed that heat treatment, such as that found in sauna therapy, was therapeutically useful in PAH patients [296]. Sauna therapy is thought to act by raising the level of the rate limiting enzyme in BH4 synthesis, GTP cyclohydrolase I, and therefore raising BH4 availability [297].

While none of these studies can be taken as definitive, they suggest that raising BH4 levels is likely to be useful in PAH therapy.

**7.9. NMDA Activity?** Evidence suggesting excessive activity of the NMDA receptors in PHA, discussed above such a mechanism in case initiation by endotoxin exposure, elevated homocysteine or paraquat exposure, exacerbation by ammonia, or the evidence, also discussed above, showing that ET-1 raised NMDA activity. Each of these observations are susceptible to various interpretations, however. Similarly, while there have been no studies aimed at testing the efficacy of agents lowering NMDA activity in PAH, five studies have shown the value of raising magnesium levels in PAH [298–303]. The NMDA receptors are highly susceptible to being activated in conditions of even marginal magnesium deficiency, so an attractive interpretation of the magnesium studies is that magnesium is acting by lowering NMDA activity. Other interpretations are possible here, as well. Clearly, this is the part of the cycle that is most weakly linked to PAH and it may be argued that it may not have a causal role at all.

## 8. Is Resveratrol a Magic Bullet for the Treatment of PAH?

Some very exciting research has shown that an agent that gives very good clinical responses in preventing PAH acts to down-regulate most of the  $\text{NO/ONOO}^-$  cycle elements.

This research was performed by Csiszar, Ungvari, and their colleagues at New York Medical College. In one study [304], they showed the following  $\text{NO/ONOO}^-$  cycle elements were elevated in PAH: oxidative stress, the inflammatory cytokines IL-6 and  $\text{TNF-}\alpha$ , other inflammatory genes,  $\text{NF-}\kappa\text{B}$  and iNOS induction. All of these were blocked along with the PAH by resveratrol, the stilbene flavonoid reported to be a wide ranging health-promoting agent. In another study [305] resveratrol prevented PAH development along with elevation of three cytokines, oxidative stress, endothelial dysfunction, and upregulation of NADPH oxidase, one of the mechanisms that generates superoxide. In a third study [306], this one on coronary arterial endothelial cells, resveratrol improved mitochondrial function, increased the mitochondrial SOD (an enzyme that gets rid of superoxide), increased reduced glutathione (an important antioxidant mechanism), lowered oxidative stress, lowered mitochondrial production of reactive oxygen species, all favorable changes lowering  $\text{NO/ONOO}^-$  cycle elements. A study by another research group [307] showed that resveratrol increased mitochondrial biogenesis and lowered angiotensin-II-dependent activity, thus providing improvements via two distinct mechanisms. Another group [308] showed that resveratrol increases synthesis of all three superoxide dismutases (not just the mitochondrial enzyme), lowered NADPH oxidase (an important source of superoxide), lowered superoxide (not surprisingly), lowered oxidative stress, and lowered  $\text{ONOO}^-$ . Most importantly, they also showed that the synthesis of BH4 was increased by inducing increases levels of the enzyme GTP cyclohydrolase I, the rate limiting enzyme in the *de novo* pathway for BH4 synthesis. Not surprisingly, the increased BH4 led to increased coupling of the eNOS enzyme [308]. Other studies have shown that resveratrol decreases the activity of the NMDA receptors and also the related kainate receptors, acting on both the receptor activities themselves [309–311] and also acting to increase glutamate transport [312], thus lowering the extracellular glutamate that acts as an NMDA agonist. In each of these actions, resveratrol is thought to act by stimulating the activity of SIRT1, a protein that has a wide ranging activity in regulating gene expression. All of this suggests that resveratrol is almost a dream agent for lowering the  $\text{NO/ONOO}^-$  cycle. By raising SIRT1, it lowers, at least in some tissues each of the following  $\text{NO/ONOO}^-$  cycle elements: oxidative stress,  $\text{ONOO}^-$ , mitochondrial dysfunction, superoxide, inflammatory cytokines and other inflammatory markers,  $\text{NF-}\kappa\text{B}$  and excessive NMDA activity while raising BH4 levels. Almost the whole cycle is lowered by resveratrol.

Should we be surprised that another research group asked [313]: is resveratrol the magic bullet for pulmonary hypertension?

It is this author's view that resveratrol is likely to be a useful therapeutic agent for PAH but that it is not likely to be a magic bullet. The basis of this assessment is two-fold. Firstly, it is much more difficult, in general, to cure disease than to prevent it and this is to be especially expected for such a mechanism as the  $\text{NO/ONOO}^-$  cycle, whose robust structure (Figures 1(a)–1(e)) strongly argues that it is difficult to down-regulate. These studies were basically preventative

rather than curative [304–306]. Furthermore, the activity of SIRT1 [314], which mediates each of these resveratrol effects, is sensitive to oxidative inactivation and is also dependent on NAD but NAD can be massively depleted by the elevation of PARP activity by ONOO<sup>-</sup> in the NO/ONOO<sup>-</sup> cycle (see number 18 of the 34 mechanisms). It follows from this that replenishing NAD with high doses of nicotinamide and nicotinic acid (forms of niacin) will be needed as well as the use of antioxidants and lowering of ONOO<sup>-</sup>, for resveratrol to be very active. In general, the more severe the disease and the cycle, the more difficult it will be to do all that effectively.

## 9. Discussion and Conclusions

The NO/ONOO<sup>-</sup> cycle is a complex biochemical/ physiological vicious cycle that can explain various chronic inflammatory diseases localized to certain regions of the body. It should be considered as an etiologic mechanism only for such inflammatory diseases that can be initiated by stressors, including short-term stressors, that can elevate cycle elements and can, therefore, at least in principle, initiate the cycle through such elevation. Clearly PAH is such a disease and, therefore, PAH may be a candidate for such an etiology. What should be clear from this paper, is that there are many diverse studies on PAH, each implicating NO/ONOO<sup>-</sup> cycle elements in PAH (much of these are summarized in Table 2). While many of these do not show that these elements have causal roles in causing PAH, so that they may simply be an epiphenomenon simply based on those studies, there are also many studies that do show a causal role for 11 out of 12 cycle elements. These collectively therefore provide strong support for a NO/ONOO<sup>-</sup> cycle etiology.

Furthermore, the cycle is based on five principles the fit to each of which provides a very different type of evidence for the causality of the cycle. In PAH, there is strong evidence that stressors that initiate cases of PAH elevate cycle elements, showing the each of these initiators can potentially act to initiate the cycle in this way (Principle 1), see Table 1. There is strong evidence that most of the elements of the cycle are elevated in the chronic phase of the disease (Principle 2). There is strong evidence that the symptoms and signs of PAH can be produced by cycle elements (Principle 3), provided in the introduction and elsewhere in this paper. There is also strong evidence for a local mechanism in PAH, localized to the pulmonary arteries (Principle 4), also discussed in the introduction. In addition, there is also strong evidence, mostly derived from animal model studies, that agents that lower cycle elements can be useful in therapy (Principle 5).

However, this does not mean that there are no weaknesses in the case. The weakest part of the argument is that there is no direct evidence that excessive NMDA activity in the pulmonary arteries have a role. There is evidence for the existence of NMDA receptors in the pulmonary arteries. There is evidence that two stressors that elevate NMDA activity, high levels of both homocysteine and ammonia, each have roles in causing PAH. However, there have been no studies to test whether the NMDA receptors have roles in these or other observations about PAH.

With regard to causal roles in PAH, the following elements of the cycle appear to have causal roles, as documented in various parts of this paper.

- (1) From the introduction, elevated ONOO<sup>-</sup>, consequent oxidative stress, inflammatory responses, and mitochondrial dysfunction all have causal roles in generating the symptoms and signs of PAH.
- (2) From the subsection on RhoA elevation, NF- $\kappa$ B elevation, inflammatory cytokines, and oxidative stress all have causal roles in elevating RhoA.
- (3) From other sections on initiation of cases of PAH by initiating stressors, BH4 depletion, oxidative stress, two of the TRP receptors, and one of the inflammatory cytokines, all have causal roles. Independent of the issue of causality, each of the initiators, as seen in Table 1, elevate, multiple cycle elements.
- (4) From the section on Endothelin-1 (ET-1), the following have causal roles in raising ET-1 levels: NF- $\kappa$ B, oxidative stress, inflammatory cytokines, and one of the TRP receptors (TRPV1).
- (5) Finally, from the section on elevated cycle elements in the chronic phase of disease and also therapy via agents that lower cycle elements 10 of the 12 cycle elements have causal roles based on therapy and 11 of the 12 have causal roles based on other types of evidence. The only element not causally implicated is excessive NMDA activity where no directly relevant studies are available. There are, in addition many other studies where these cycle elements are implicated but where causality is uncertain. It is difficult to see how that could all be true unless the NO/ONOO<sup>-</sup> cycle or something similar to it is the central cause of PAH.

There is one additional consideration with regard to these data. Each of the individual observations summarized earlier in this Discussion and Conclusions Section can be interpreted in various ways. It is the pattern of evidence that argues for a NO/ONOO<sup>-</sup> cycle etiology, not the individual studies or observations. And in most cases, this is true of other proposed NO/ONOO<sup>-</sup> cycle diseases as well—it is only the pattern of evidence that makes a convincing case. However in PAH there are two exceptions to this and they relate to biological components that have causal roles in PAH.

As discussed in the Endothelin-1 (ET-1) section, there is strong evidence that ET-1 has a causal role in PAH. The only way that ET-1 can have a causal role if PAH is a NO/ONOO<sup>-</sup> cycle disease, is if ET-1 is behaving as part of the cycle in the pulmonary arteries. That is, ET-1 must be synthesized in increasing amounts in response to cycle elements and must in turn increase cycle elements. These are, then, strong predictions of the NO/ONOO<sup>-</sup> cycle mechanism, if it applies to PAH, predictions that are not likely to be made based on any other hypothesis. So are both of the predictions true? Evidence was already summarized in 4 above that ET-1 is synthesized in response to four cycle elements, NF- $\kappa$ B oxidative stress, inflammatory cytokines, and one of the TRP receptors (TRPV1). So the first prediction is clearly

TABLE 2: Summary of evidence regarding roles of NO/ONOO<sup>-</sup> cycle elements in chronic phase of pulmonary arterial hypertension (PAH).

NO/ONOO <sup>-</sup> cycle element	Elevated?	Response to possible therapeutic agent aimed at lowering?	Other causal evidence?
Oxidative stress	Yes	Yes	Yes
superoxide	Yes	Yes	Yes
ONOO <sup>-</sup>	Yes	Yes	Yes
NO	?	? only evidence comes from iNOS studies	Yes
NF-κB	Yes	Yes	Yes
Inflamm. cytokines	Yes	Yes	Yes
iNOS	Yes	Yes	Yes
Mitochondr. dysfunct	Yes	Yes, limited to PARP studies	Yes
TRP receptors	Yes	Yes	Yes
Intracellular Ca <sup>2+</sup>	Yes	Yes	Yes
BH4 depletion	Yes	Yes	Yes
NMDA recept activity	?	?	No

The other causal evidence column is limited to either genetic studies or studies, where the element has been reported to have a role in the generation of symptoms and/or signs of PAH.

supported. Going back to the Endothelin-1 section, it can be seen that ET-1 acts, in turn, to elevate the following elements of the cycle: oxidative stress, superoxide, the ONOO<sup>-</sup> cycle marker 3-nitrotyrosine, NF-κB activity, BH4 depletion, iNOS induction, TRP receptor function, intracellular calcium levels, mitochondrial dysfunction, NMDA activity, and inflammatory cytokines. Essentially the whole cycle is elevated in response to ET-1. I must note, that some of these studies have been done not in the context of PAH but rather in other regions of the body where ET-1 is also active, including regions of the brain. Nevertheless, they provide about the closest thing to smoking gun evidence that one is likely to find, confirming specific predictions of a NO/ONOO<sup>-</sup> cycle etiology for PAH.

There is a similar, albeit somewhat weaker, case for a causal role for RhoA as a causal factor in PAH acting as a NO/ONOO<sup>-</sup> cycle element. Three cycle elements act to raise RhoA, as seen in 2 above. RhoA acts in turn, as shown in that section of the paper, to elevate superoxide, ONOO<sup>-</sup>, oxidative stress, BH4 depletion and intracellular calcium. So here we have a similar, albeit slightly weaker example of smoking gun evidence for a NO/ONOO<sup>-</sup> cycle etiology for PAH.

It may be concluded that it is highly probable that PAH is a NO/ONOO<sup>-</sup> cycle disease, with the only substantial weakness being the lack of direct evidence for a causal role for excessive NMDA activity in PAH.

This substantial weakness provides a simple test of the NO/ONOO<sup>-</sup> cycle mechanism for PAH. The cycle mechanism predicts that excessive NMDA activity has an important causal role in PAH and that this role may be particularly important in initiation via mechanisms such as hypoxia, homocysteine, or endotoxin, which, as discussed above, may act in part via excessive NMDA activity. These are easily testable predictions and should be tested in my no doubt somewhat biased view, possibly by using such relatively

well-tolerated NMDA antagonists such as memantine or dextromethorphan.

An inference from the NO/ONOO<sup>-</sup> cycle mechanism for PAH is that it is very important to research multiagent protocols for PAH treatment, with agents acting to down-regulate different parts of the cycle. The robust nature of the cycle, as seen in Figures 1(a)–1(e) strongly suggest that such multiagent protocols may be the only approach to get a good sustained clinical response and possibly a cure.

The NO/ONOO<sup>-</sup> cycle concept comes from a group of previously unexplained diseases including chronic fatigue syndrome, multiple-chemical sensitivity, and fibromyalgia and also from the 34 well-accepted mechanisms listed and documented in the third section of this paper. In science, it is always important to distinguish data that were used to formulate a theory from other data that can be used to independently test it. Clearly one way of doing this for the NO/ONOO<sup>-</sup> cycle as a possibly widely applicable disease paradigm is to determine how well other diseases fit the predictions of this theory. Because of the excellent fit of PAH, it joins tinnitus [11] as an important independent test of the NO/ONOO<sup>-</sup> cycle theory of disease.

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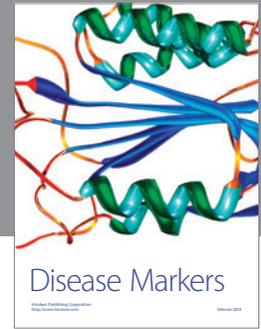
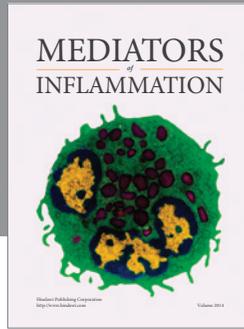
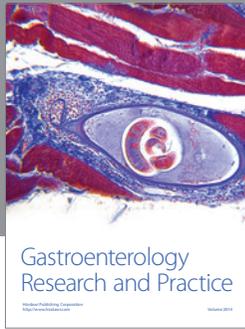
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