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Pulmonary Hypertension: Pathophysiology Beyond the Lung

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Abstract

Pulmonary hypertension (PH) is classically considered a disease of pulmonary vasculature which has been the predominant target for drug development and PH therapy. Despite significant advancement in recent years in identification of new drug targets and innovative treatment strategies, the prognosis of PH remains poor, with median survival of 5 years. Recent studies have demonstrated involvement of neuroinflammation, altered autonomic and gastrointestinal functions and increased trafficking of bone marrow-derived cells in cardiopulmonary pathophysiology. This has led to the proposal that PH could be considered a systemic disease involving complex interactions among many organs. Our objectives in this review is to summarize evidence for the involvement of the brain, bone marrow and gut in PH pathophysiology. Then, to synthesize all evidence supporting a brain-gut-lung interaction hypothesis for consideration in PH pathophysiology and finally to summarize unanswered questions and future directions to move this novel concept forward. This forward-thinking view, if proven by further experiments, would provide new opportunities and novel targets for the control and treatment of PH.

Graphical Abstract

In a novel view of pulmonary hypertension (PH) pathophysiology as a systemic disease, a growing body of evidence indicates that bone marrow-derived cells, gut and brain are also involved in PH development and progression. PH risk factors stimulate autonomic brain areas, initiating altered neuron-microglia communication, leading to increased activation of sympathetic nervous system (SNS) that : (I) in the lungs induces a series of well-established signaling events associated with pulmonary pathophysiology; (II) mobilizes *bone marrow (BM)* progenitor cells that besides contributing to pathophysiological changes in the heart and lungs, may also migrate to other organs such as brain and gut, contributing to the persistent neuroinflammation, altered neuron-microglia communication and gut inflammation observed in animal models of PH and PAH.

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Keywords

Pulmonary hypertension; Neuroinflammation; Bone-Marrow; Gut Inflammation; Dysbiosis and RAS

1. Introduction

Pulmonary hypertension (PH) is a progressive and devastating disease characterized by an elevation of pulmonary arterial pressure over 25mmHg, leading to right ventricular failure and premature death. First described as ‘pulmonary vascular sclerosis’ by the German physician Ernst Von Romberg in 1891, the pathophysiology of pulmonary hypertension is still incompletely understood. The World Health Organization (WHO) described and classified the known causes of PH by group: (1) Pulmonary arterial hypertension (PAH); including idiopathic (IPAH), heritable, drug/toxin induced or associated with other systemic diseases as HIV, lupus and schistosomiasis; (2) PH due to left heart disease; (3) PH due to chronic lung disease and/or hypoxemia; (4) PH due to thromboembolic disease; (5) PH due to unclear multifactorial mechanisms (113).

Patients diagnosed with pulmonary hypertension describe their most common symptoms as shortness of breath (dyspnea), fatigue, chest pain, heart palpitations, higher susceptibility to respiratory infections, fluid retention, and cognitive effects such as anxiety and depression. Besides being a debilitating disease, pulmonary hypertension has a poor prognosis, is often diagnosed late in the disease process and lacks effective treatment, all of which highly

impact the daily life of these patients. Ginoux *et al.* recently published a study conducted in France that evaluated 248 patients in groups 3 or 4 of pulmonary hypertension. The median survival was only 46 months and the prognosis was even worse in the elderly (> 65 years) and very elderly patients (> 75 years) being 37 months and 28 months, respectively (38). Despite great efforts to introduce new therapies, some patients are refractory to multidrug therapy and lung transplantation is the only available option (36). This emphasizes the continued need for more effective medications and further research.

This review aims to present the view of pulmonary hypertension as a systemic disease and the need for further research to better understand the complexity of pulmonary hypertension pathophysiology and the different targets that could be approached for better outcomes. A systemic approach might allow the production of safer and more effective drugs that might be used in pre-symptomatic patients, or during the management of severe pulmonary hypertension, especially for those patients who do not respond to current medical therapy.

2. Pathophysiology

2.1 Heart

Whatever the cause, increased resistance of the pulmonary vasculature results in increased right ventricular systolic pressure to preserve cardiac output. Chronically, progressive increases in resistance of the pulmonary vasculature impose increased afterload on the right ventricle inducing an increase in right ventricular systolic pressure (RVSP), followed by increased wall stress, impaired right coronary artery flow and increased oxygen demand. This impairment of oxygen supply and demand along with higher afterload has been implicated in the development of the right ventricular myocardial hypertrophy (RVH) observed in PH patients and is a common compensatory mechanism in PH (37, 103).

The classic view of progression of PH assumes that the heart failure is a consequence of elevated afterload. However, Bogaard *et al.* using two animal models of progressively increasing pulmonary arterial pressure, pulmonary artery banding and Sugen-Hypoxia (combined exposure to the vascular endothelium growth factor (VEGF) receptor blocker SU5416 and hypoxia), demonstrated that only the Sugen-Hypoxia model induced heart failure (HF). Thus, increased pulmonary arterial pressure was insufficient to induce right HF (RHF). Currently, little is known about the progression from compensated RVH to decompensated right ventricular failure in PH (11, 103).

Arterial remodeling of the pulmonary vasculature is important in PAH (26, 56, 77). It includes smooth muscle cell proliferation, endothelial injury, enhanced extracellular matrix deposition associated with chronic inflammation and increased circulating levels of inflammatory cytokines and chemokines. The enhanced release of mediators from the remodeled vasculature in PAH may contribute to compensated/decompensated RVH (25). Further, sympathetic activation has been related to progression of heart failure and is a predictor of severe deterioration in patients with PAH (18). Another component that may contribute to RHF is the increased trafficking of bone marrow-derived cells to the heart, contributing to hypertrophy and perivascular remodeling (see section 1.2) (28). A better understanding of this process may be extremely important for PH management because,

rather than pulmonary stenosis/remodeling, the most important predictors of deleterious PAH effects relate to right ventricular function (18, 37, 65). This was demonstrated by Ghio et al. who found that patients with high pulmonary arterial pressure and low right ventricular ejection fractions (RVEF) had 4.3 times worse prognoses compared to patients with preserved RVEF. While high risk of death and urgent transplantation was observed in patients with low RVEF, the prognosis for PH patients with preserved RVEF was similar to patients with normal pulmonary arterial pressure (37). Furthermore, Tonelli et al. studied the cause and circumstances of death in PAH with a cohort of 132 patients. These subjects were Functional Class III or IV at the time of the last visit by New York Heart Association classification. 93% of the 87 patients in PH group I had physical findings suggestive of right heart failure and 76% of them died of right heart failure. The remainder died of other causes (121).

2.2 Bone Marrow and Inflammation

Several lines of investigation suggest that some of the cells involved in remodeling and perivascular infiltration in pulmonary hypertension are bone marrow-derived cells (5, 10, 17, 28, 33, 61, 75, 83). Experiments with eGFP-BM-transplanted mice, showed that PH increased bone marrow-derived cell trafficking into the pulmonary parenchyma and these cells were involved in remodeling and perivascular infiltration in the lungs (10). Similar infiltration of bone marrow-derived cells was also observed in the heart. These cells differentiate either into cardiomyocytes or if located perivascularly into myofibroblasts, to contribute to cardiac hypertrophy or perivascular fibrosis respectively (28).

Mutations in the coding region of bone morphogenic protein receptor 2 (BMPR2) have been observed in 70% of patients with hereditary PAH (19, 67, 96) and caveolin mutations have been associated with further proliferation and cell migration also contributing to PAH pathophysiology (43, 52, 70). Asosingh *et al.* isolated bone marrow-derived CD133 (+) cells from patients diagnosed with PAH caused by BMPR2 or caveolin mutations and healthy subjects (4). Transplantation of the cells from PAH patients, but not healthy subjects, induced right heart hypertrophy and angiogenic remodeling in immunodeficient NOD-SCID irradiated mice. Furthermore, bone marrow-derived proangiogenic cells (PACs) directly contribute to small-vessel remodeling in PH; their ablation in the Sugen-Hypoxia model reduced RVSP, inhibited the muscularization and stiffening of pulmonary arterial vessels with no difference in RV remodeling (10). According to these authors, serotonin is the key for function and recruitment of PACs in PAH, as lack of 5-hydroxytryptamine receptor 2B (5-HT_{2B}), also known as serotonin receptor 2B, showed similar protective outcomes. Alternatively, Guignabert *et al.* demonstrated that fluoxetine, an inhibitor of the serotonin reuptake transporter (5-HTT), but not specific antagonists of the serotonin receptors, 5-HT_{1D/1B}, 5-HT_{2A} or 5-HT_{2B}, reversed severe PH or completely inhibited monocrotaline-induced PH (41). Besides recruiting bone marrow-derived cells to the lung vasculature, it is well known that serotonin synthesized in endothelial cells of pulmonary arteries induces vasoconstriction and remodeling of pulmonary artery smooth muscle cells and fibroblasts via paracrine signaling (24).

On the other hand, a protective effect of BM-derived mesenchymal stem cells (MSC) has been explored in regeneration of myocardial tissue after acute myocardial infarction. BM-MSCs differentiated into cardiomyocytes in the injured myocardium, reduced post-MI remodeling and improved cardiac function (65, 76, 114, 120). Further, endothelial progenitor cell therapy and mesenchymal stem cell autologous transplantation attenuated the effects of monocrotaline in rats. However, according to the Pulmonary Hypertension Association, “Stem cell use has not been adequately studied and neither the efficacy nor safety of stem cells to treat PAH has been established”. These conflicting outcomes indicate that more investigation is needed to better understand the role of bone marrow-derived cells to cause or to protect against pulmonary hypertension.

Some questions that remain unanswered about this phenomenon are: 1. Is bone marrow-derived cell trafficking exclusive to heart and lung parenchyma or are other organs also targeted by these cells to contribute to PH progression as a systemic disease? 2. What is the mechanism involved? 3. Is the trigger for changes in the composition of bone marrow cells in tissues a direct regulation of bone marrow inflammatory cell activity by the autonomic nervous system or is it mediated by cytokines? 4. Is it mediated by a specific cell type? 5. Would alteration of the BM cell populations in tissues by autologous stem cell transplantation be a potential new therapy for PH patients?

2.3 Gut inflammation and Microbiota

The U.S. Food and Drug Administration (FDA) reported gut disorders in PAH patients including diarrhea, constipation, nausea, etc. These symptoms are currently considered side effects of treatment by most physicians and researchers (1). However, in a controlled clinical trial for sildenafil, GI issues such as nausea and vomiting and abdominal discomfort were alleviated in the treatment arm, but constipation was worse in the placebo arm, suggesting GI issues severe enough for some patients with PH to describe (101). This indicates that these gut disorders might be unrelated to side effects of treatment but rather part of a cascade of events in the syndrome of PH. Unfortunately, besides descriptions by patients, no further evaluation of the origin of gut disorders in pulmonary arterial hypertension patients has been attempted.

Animal models of PH such as monocrotaline (MCT) or chronic hypoxia exposure, that mimic the PH classified as group 3 by the WHO, are related to chronic lung disease. In both models, we have observed histopathological signs of gut inflammation such as shorter villi, decreased goblet cells, increased deposition of extracellular matrix and fibrosis (unpublished data). These occur in the absence of other treatments that could have side effects on the gut. Our group has also detected significant increases in plasma biomarkers for gut leakiness [zonulin and Intestinal fatty-acid binding protein (iFABP)] and gut inflammation [lipopolysaccharides (LPS), high mobility group box 1 (HMGB1) and tissue inhibitor of metalloproteinase 1 (TIMP1)] in PAH patients indicating gut inflammation and increased permeability is present in PAH patients (39). This suggested the hypothesis that PH is associated with increased gut permeability, which may cause microbial translocation and contribute to chronic inflammation.

Gut microbiomes differ between individuals, however, healthy individuals share similar profiles that differ from unhealthy individuals. The goal of gut microbiome research is to identify the differences between good health and illness and more specifically identify bacterial species that are markers of specific disease. Dysbiosis is the term to describe imbalance of gut microbial composition and has been described in several diseases. The influence of gut microbiota in these diseases became even clearer with experiments using fecal matter transplantation (FMT). The gut microbiome of patients with depression is not only different from subjects without depression, but when transferred to germ-free mice induced depressive behavior. Similar experiments performed with FMT from hypertensive patients caused an increase in blood pressure, emphasizing the importance of a balanced microbial community in the intestine to homeostasis (2, 40). The influence of gut microbiota in pathogenesis and progression of chronic lung diseases such as asthma, respiratory infections and chronic obstructive pulmonary disease (COPD) has also been described (27, 45, 47, 69, 71, 86, 87, 99, 115, 124, 125).

Recently, Callejo *et al.* demonstrated that PAH induced gut dysbiosis. Even in early phases of PAH induced by Sugen-hypoxia in rats, a significant increase in Firmicutes/Bacteroidetes (F/B) ratio was observed, a recognized biomarker of dysbiosis (14). A comparison of these findings by Callejo *et al.* to our own reveals certain parallels between Sugen-hypoxia PAH and hypoxia-induced PH that may be key contributors to PH pathophysiology, i.e.: (1) Increased sympathetic activity (LF/HF) (88, 105); (2) Gut inflammation; (3) 95% of total serotonin in the body is synthesized in the gut in healthy individuals, and serotonin pathways are dysregulated in functional GI disorders (15). Together, these data led us to hypothesize that the chronic increase of sympathetic activity reported in PH patients (16, 102) and that we observed in rodent PH models, may induce gut inflammation and increase gut permeability leading to gut microbial dysbiosis that might influence serotonin release (5-HT) (24).

3. Central Nervous System (CNS)

3.1 Autonomic Regulation

Increasing evidence describes the contribution of autonomic imbalance to pathophysiology of pulmonary hypertension (18, 44, 82, 85, 93, 123, 127). It is well known that chronic sympathetic activation leads to cardiac hypertrophy, ventricular dysfunction, remodeling, arrhythmia and apoptosis (34, 68, 73, 74, 127). In the lungs, sympathetic stimulation contributes to basal tone via alpha-adrenoreceptor-mediated vasoconstriction since denervation causes pulmonary vasodilation (7). However, the mechanisms involved in sympathetic activation in PAH are still unclear.

Levels of circulating catecholamines in PAH patients compared to healthy controls may not be indicative of sympathetic outflow, as catecholamines are affected by degradation, metabolism, synthesis, neuronal release or reuptake (44, 82, 85, 93). Therefore, directly recording nerve traffic in muscle sympathetic nerves (MSNA) is more effective for identifying significantly higher sympathetic activity in PH patients compared to healthy individuals. Increased MSNA correlated with heart rate, oxygen saturation and exercise

impairment and was associated with clinical deterioration. Moreover, increased MSNA can be used as an independent predictor of severe deterioration in patients with PAH (18, 123).

Several reports have shown that sympatho-inhibition might be a potential alternative target to improve PH outcome. For example, pulmonary artery denervation significantly reduces pulmonary pressure and remodeling in PH, while cutting vagal connections to the pulmonary region leads to fibrosis in pulmonary arteries and airways. More recently, Yoshida *et al.* (131) used vagal nerve stimulation to restore autonomic balance, preserve right ventricular function, ameliorate vascular remodeling and improve survival in rats with severe PAH.

Alpha/beta-adrenergic receptor antagonists such as arotinolol, prevented monocrotaline-induced PH in rats (54), while carvedilol has shown encouraging results. It improved RV function, reversed established RVF in monocrotaline and Sugen-hypoxia models of PH and successfully inhibited proliferation of human vascular smooth muscle cells *in vitro* (90). Moreover, studies in PAH patients have shown that carvedilol improved heart rate recovery after exercise compared to placebo. The therapeutic potential of carvedilol in PAH is currently being investigated in phase 2 of a clinical trial ([ClinicalTrials.gov](https://clinicaltrials.gov) identifier:) (29). Although the use of beta-blockers for therapeutic treatment of cardiovascular outcomes in PH has shown promise, it needs careful evaluation, as β -blockers also have negative inotropic and chronotropic effects that might worsen stages of severe heart failure.

3.2 Chemorreflex

Dyspnea is the principal debilitating and presenting symptom of PH patients. Dyspnea is explained by alterations in pulmonary vasculature leading to abnormal gas exchange associated with impaired cardiac output and increased ventilatory drive. Control of ventilation is performed by respiratory centers in the brainstem and can be modulated by higher brain centers and systemic chemoreceptors. The chemoreflex is an important physiological mechanism that responds to decreases in PO₂ through peripheral chemoreceptors or increased PCO₂ by central chemoreceptors, as in dyspnea, with a systemic increase in sympathetic activity.

It is perhaps logical to assume that the origin of neurohumoral dysfunction in PH is positive feedback induced by dyspnea and that hyperoxia (100% O₂ exposure) would normalize sympathetic activity induced by the chemoreflex. However, Velez-Roa *et al.* demonstrated that hyperoxia only attenuated the increased MSNA in PAH patients by 25%, indicating independence from chemoreflex control (123). This opens the possibility that chronic activation of sympathoexcitatory brain areas might drive increased sympathetic activity in PH patients rather than it being simply a reflex response to lower oxygen levels.

3.2 Innate immune system

Previous reports indicate inflammation as a major contributor driving pulmonary vascular disease towards pulmonary hypertension (26, 91). In experimental PH, inflammation precedes vascular remodeling suggesting altered immunity as a cause rather than a consequence of vascular disease (119). Different populations of inflammatory cells including monocytes, macrophages, dendritic cells and T- and B- lymphocytes are found in

the vascular lesions of PH (91, 116, 119). Activation of these cells releases inflammatory cytokines and chemokines, such as IL-1 β , IL-6, IL-8, monocyte chemoattractant protein (MCP)-1, fractalkine, CCL5/RANTES and tumor necrosis factor (TNF)- α which further facilitate the cell proliferation and inflammatory processes of PH correlated with worse clinical outcomes (17, 53, 77, 102, 116, 117).

Cytokines and chemokines evoke further inflammatory cascades that induce cell proliferation, apoptosis and remodeling of the pulmonary vasculature, leading to medial hypertrophy and lumen obstruction. In response to persistent tissue injury, high mobility group box 1 (HMGB1), a proinflammatory cytokine, activates pattern recognition receptors or PRRs, including toll-like receptor 4 (TLR4). Activation of PRRs is recognized as a starting point in activation of innate immunity, inducing the inflammation process, cell recruitment and tissue remodeling. Bauer *et al.* corroborated that by showing that chronic hypoxia exposure induces a 3-fold increase in TLR4 expression in the lung of PH animals. While exogenous HMGB1 exacerbates PH pathology by TLR4 activation, TLR4 deficiency attenuates it (8, 126). In contrast, a study reported that TLR4 deficiency spontaneously led to PAH (66).

Fractalkine (CX3CL1 or FKN) is a soluble chemotactic chemokine with actions mediated by CX3 chemokine receptor 1 (CX3CR1) expressed on monocytes, macrophages, subpopulations of T lymphocytes, neurons, mast cells, natural killer cells and microglia. Upregulation of CX3CL1 in CD4+ and CD8+ T lymphocytes of PAH patients was reported (6). In addition, we and others have observed that genetic and pharmacological inactivation of CX3CR1 ameliorates pulmonary remodeling in hypoxia-induced PH (3, 88). According to Amsellem and colleagues, this protective effect could be explained by modulation of monocyte recruitment to the lungs and decreased polarization of macrophages towards the proinflammatory state leading to decreased smooth muscle cell proliferation in the pulmonary artery. An alternative or associated explanation that we espouse attributes the protection observed in CX3CR1-KO mice to the fact that this is an important pathway of neuron-microglia communication, and the lack of microglia activation observed in those animals might be crucial for this protection. Our hypothesis is further explored in the following section.

3.2.1 Microglia—Microglia are the resident innate immune cells of the CNS, the major contributor to CNS inflammation or neuroinflammation, and have been recognized as an important component of neurodegenerative disease and neuronal dysfunction (58). In healthy brains, microglia are so dynamic that it is estimated that the entire brain of mice is scanned by microglia every hour, influencing synaptic connections, activity and neuronal health (58, 84). Microglia are key modulators of the immune response in the brain and can be activated very early in pathology, migrating to the injury site to phagocytose dying cells or debris, and to produce and release neurotoxic and protective molecules. Microglia are frequently described by their morphology; “resting” when highly ramified and performing brain surveillance and “activated” when they have shorter processes and a larger cell body. While different forms of microglia activation lead to different activation pathways, classically activated microglia have increased expression of Iba1, increased release of pro-inflammatory cytokines and are involved in neuroinflammation (105).

As the most dynamic cells in the CNS, microglia have highly motile projections and rapid responses to ATP, indicating that they can migrate to the most active areas in the brain (22). Microglia motility is also enhanced by serotonin, IL-4, IL-8, MCP-1 and VEGF, showing the need for further investigation to determine the chemotactic factors specific to different pathologies (59, 64, 72). Microglia can interact with neurons electrically, chemically and physically, modulating neuronal responses including regulation of sympathetic activity (Figure 1) (58). Taken together, this leads us to consider that chronic stimulation of neurons might induce activation of microglia. Further, this increased interaction between microglia and neurons might stimulate the increase in sympathetic activity observed in PH patients (and animal models of PH) that predicts worsening PH prognosis and severity.

Studies have shown that glia can also influence neuronal activity, leading to modulation of respiratory responses. During the first hour of exposure to chronic hypoxia microglial activation is induced in the nucleus tractus solitarius (NTS), an integration center for peripheral chemoreceptors that contributes to respiratory control. Inhibition of microglia activation with minocycline resulted in absence of ventilatory acclimatization to hypoxia. Inhibition of this normal physiological response to hypoxia by minocycline, indicates that microglial cells modulate respiratory responses (118). Interestingly, exposure to LPS or hypoxia during gestation induces an enhanced response to re-exposure of microglia in vitro, suggesting that microglial “remember” prior exposures (16, 55).

Recently, our group unveiled microglial activation in the paraventricular nucleus of hypothalamus (PVN) induced by monocrotaline treatment or chronic hypoxia models of PH. The PVN has emerged as one of the most important centers of autonomic control in the brain (20, 30, 88, 97, 105). An explosion of new findings has increased our understanding that microglia mediate tissue homeostasis in health and disease. However, the mechanism of microglial activation and how it is involved in disease processes remains elusive.

One of the pathways that may explain neuron-microglia interaction is the CX3CR1-CX3CL1 axis. The neuron-derived chemokine, CX3CL1, regulates microglial activation by interaction with its receptor, CX3CR1, expressed on microglia. Microglia in CX3CR1-deficient mice have a different morphology compared to those of wild type mice and fail to modify in response to environmental effectors, remaining set in a “resting state” (98). Remarkably, CX3CR1-KO mice are protected from hypoxia-induced PH. This was associated with blunted enhancement of microglial cell number and activation in the PVN that is usually observed upon exposure to chronic hypoxia. Furthermore, RVSP, a surrogate of pulmonary pressure, positively correlated with the number of microglia in the PVN (3, 88).

We also demonstrated that intracerebroventricular (ICV) infusion of minocycline into monocrotaline-treated rats ameliorated pulmonary vascular remodeling, blunted the increase in RVSP, reduced right ventricular fibrosis and hypertrophy and prevented sympathetic activation induced by monocrotaline. This effect was accompanied by decreased numbers of microglia in the PVN, decreased microglial activation and consequently prevention of the increase of inflammatory cytokines such as TNF- α , IL-1 β , IL-6 and decreased IL-10

observed in monocrotaline-treated rats (105). This was a central effect because the effective icv minocycline dose was ineffective when given via peripheral infusion.

Another hypothesis that has been investigated is the involvement of serotonin in the development of PH. Blockade of serotonin receptors has shown potential benefits for the management of pulmonary hypertension outcomes, and even reversed severe pulmonary hypertension induced by monocrotaline. Researchers have extensively debated the mechanism responsible for those findings. Some argue that it may be related to the antagonism of direct effects of serotonin in the pulmonary vasculature, while others suggest the mechanism is antagonism of recruitment of bone marrow-derived cells to the lung and heart, as discussed in the section 2.2. We otherwise suggest that it may be a combination of those effects, associated with decreased recruitment of bone marrow-derived cells to PVN. The recruited cells may contribute to neuroinflammation observed in this nucleus and consequently to unbalanced autonomic activity. Furthermore, serotonin itself has been related to increased activation of PVN neurons that may also contribute to neuroendocrine responses in PH (51).

Recent studies have correlated depression, a very common symptom in PH patients, to increased levels of circulating pro-inflammatory cytokines and microglial activation (48, 62, 92, 128). Pro-inflammatory cytokines positively correlated with depression score index in a study of 92 patients diagnosed with lung cancer (62). Those findings are also consistent with new advances in our understanding of systemic hypertension, particularly treatment-resistant hypertension. Recent studies have demonstrated that in hypertension models such as angiotensin II (Ang II)-infusion, PVN pre-autonomic neurons activate, increasing sympathetic activity, and releasing inflammatory cells from the bone marrow. The inflammatory bone marrow cells consequently migrate to the vasculature, to contribute to vascular pathology, and to the PVN, where they differentiate into BM-derived microglia-like cells (100, 133, 134). We have also shown that Ang II induces changes in gut microbiota and gut pathology that are ameliorated by microglia inhibition (107). This indicates that in treatment-resistant hypertension, microglial activation is involved in sustained sympathoexcitation which results in perpetuation of high blood pressure, gut inflammation, increased release of BM-derived cells (106).

Collectively, this information suggested that our group should investigate the contribution of neuroinflammation and sympathetic activity in PAH models. We have published a growing body of data indicating a critical role of microglial activation and neuro-inflammation in autonomic brain regions in pulmonary hypertension (PH), possibly resulting in increased sympathetic activity (50, 88, 105). This may appear contradictory, as the neuroinflammation and microglia activation in the PVN and increased sympathetic activity observed in systemic hypertension have been correlated with increased blood pressure and ultimately left heart failure. Also, the neural substrates involved are common, such as the location of baroreceptors and chemoreceptors, having the NTS as the site of the first afferent synapse and signals from baroreceptors and chemoreceptors ultimately converging on the same neurons in the rostroventrolateral medulla (RVLM). However, although blockade of glutamate receptors in the intermediate ventrolateral medulla (IVLM) inhibited the baroreceptor reflex, the chemoreflex was unaffected (21). This indicates that while the

pathways involved in cardiovascular and respiratory control may have some similarities, they actually have distinct pathways that moreover, result in different outcomes.

New emerging technologies such as single cell RNAseq have brought to light the existence of several subtypes of microglia, with different gene expression profiles that depend upon their location in the brain and state of health or disease, indicating that these subtypes of microglia may have alternate functions in health and disease states (81, 122). These techniques open the opportunity not only for a better understanding of microglia involvement in various pathologies but also might unmask potential biomarkers and new targets for future personalized therapies.

4. Renin-angiotensin-system (RAS)

Substantial evidence supports chronic activation of RAS in the pathophysiology of PH (23, 49, 89). The vasoconstrictive/vasodeleterious axis comprised by angiotensin converting enzyme (ACE), angiotensin II (AngII) and the angiotensin type 1 receptor (AT1R) is upregulated in patients and in animal models of PH. Also, De Man *et al.* reported that increased angiotensin I (Ang I) and AngII correlated with mortality in 58 patients with idiopathic PAH (iPAH) (23). The activation of the ACE-AngII-AT1R axis increases inflammation, oxidative stress, and has pro-thrombotic, hypertrophic, and pro-fibrotic effects. In PH, ACE activity is 50% higher in pulmonary vessels and ~4 fold higher in the right ventricle after 14 days of chronic hypoxia exposure, exacerbating the remodeling in the pulmonary vessels and cardiac hypertrophy in rats. Whereas blockade of this axis with the AT1R antagonist, losartan or the ACE inhibitor captopril was beneficial, improving hemodynamics in MCT and hypoxia-induced PH models, its clinical use had mixed results and failed to successfully manage PH. (23, 78–80)

The imbalance of RAS may also contribute to systemic changes observed in pulmonary hypertension. A single dose of Ang II induces activation of microglia and activates neurons and the immune system (134). Also, studies from Haznedaro lu *et al.* revealed that RAS can be locally activated in the bone marrow affecting growth, production and differentiation of hematopoietic cells (46).

Conversely, ACE2, an enzyme highly expressed in the lungs, catalyzes the conversion of AngII to Ang-(1–7), a peptide that activates the Mas receptor and counteracts the deleterious actions of ACE-AngII-AT1R axis. To date, downregulation of ACE2 and Ang1–7 have been reported in patients with pulmonary hypertension. This suggests that another strategy to target the RAS imbalance observed in PH would be to counteract the effects of the ACE/AngII/AT1 axis by activating the vasoprotective ACE2/Ang1–7/Mas axis. A growing body of evidence has shown that increasing ACE2 activation using various tools ameliorates pulmonary hypertension outcomes (12, 13, 31, 32, 42, 49, 60, 95, 108–111, 129, 132). Preclinical studies showed that a single dose of recombinant human ACE2 improved cardiac output and pulmonary vascular resistance in PAH patients (43) and is currently under clinical trial ([ClinicalTrials.gov](https://clinicaltrials.gov) identifier:)(60, 108, 109, 129, 132).

It is important to highlight some findings to better understand the complexity of RAS influence in the pathophysiology of PH: (1) Our group has shown the protective effect of enhancement of ACE2/Ang-(1–7)/Mas axis activity in pulmonary hypertension (12, 31, 32, 95, 108–111, 129). Diminazene (DIZE), an ACE2 activator, besides preventing and reversing PH in various animal models of PH, also restored migratory capacity of CD34+ cells isolated from PH patients in vitro (109). Further, Liu *et al.*, recently published that microvesicles derived from mesenchymal stem cells ameliorate pulmonary hypertension by upregulation of ACE2 and Ang-(1–7) levels, as blockade of the Mas receptor with its antagonist, A-779, blunted the protective effects previously observed(63); (2) We have also observed in preliminary studies that mice overexpressing angiotensin converting enzyme 2 (ACE2KI) are protected from hypoxia-induced PH. They exhibit no neuroinflammation or microglial activation, and blunted hypoxia-induced increases in sympathetic activation; they also have a different gut microbiome composition even under normoxia (94). Henceforth, it is important to consider that besides the direct effect of RAS in pulmonary arterial remodeling and right ventricle hypertrophy, RAS imbalance has been shown to influence the function of bone marrow cells, gut microbiome composition, neuroinflammation and sympathetic activity.

5. PH as a systemic disease

Regardless of the cause, PH is a complex disease with poor outcomes that is difficult to manage. In combination, accumulated evidence of the past decades and our more recent observations question the view of PH being exclusively a lung disease. An irrefutable argument in support of this might be made in light of the reoccurrence of PH after bilateral lung transplantation. In one of the reported cases, a 62-year-old Caucasian man who underwent bilateral lung transplant for idiopathic PAH (IPAH), started to experience increasing exertional dyspnea and hypoxemic respiratory failure and was diagnosed with pulmonary hypertension after right heart catheterization. The etiology for recurrence was evaluated but unrevealing and the patient died thirteen months after his lung transplant. Histologic analysis revealed pulmonary hypertensive vasculopathy similar to that in his own lungs that were removed during the lung transplant; mutational and chromosomal analysis were also unrevealing (130). Although we only have some reported cases and it seems to be rare, the fact that it occurred highlights the relevance of evaluating the contribution of factors outside the lungs to development of pulmonary hypertension.

Monogenic causes of PH, such as BMPR2 mutations, initially generate disease at the level of pulmonary microvasculature by inducing smooth muscle cell proliferation, increased pulmonary vascular resistance and release of inflammatory cytokines into the circulation. However, the mechanism of PH pathology associated with common risk factors (environmental insults, hypoxia, etc.) may be more complex and likely involve autonomic regulation. Physiologically, the brain constantly monitors all changes in the body and responds via the autonomic system to counteract offsetting changes and keeping the body in homeostasis. Taken together, we propose the following hypothesis for consideration: We believe chronically higher neuronal activation in autonomic nuclei induces microglial activation and neuroinflammation. Increased neuronal-microglia interactions lead to an imbalance in autonomic control, with substantial increase in the SNS. This keeps the body in

“fight or flight” mode which has been associated with progression and worsening of cardiorespiratory diseases, gut inflammation and dysbiosis. It also influences the recruitment of bone marrow-derived cells to the lung, heart and possibly to brain and gut, which may further increase the neuroinflammation and gut dysbiosis that contribute to pulmonary hypertension (Figure 2). This hypothesis is supported by the findings that: (1) Hypoxia induces activation of autonomic nuclei and sympathetic activity (2) Microglial activation in autonomic nuclei is observed within hours of hypoxia exposure; (3) Microglia are responsible for innate immune memory (9), adapting subsequent responses according to previous exposures to stimuli. This may explain how effects of hypoxia and environmental insults accumulate to result in neuroinflammation; (4) Microglia modulate neuronal responses including sympathetic activity; (5) Increased sympathetic activity is correlated with progression and severity of PH; (6) Central application of minocycline prevents microglial activation, attenuates SNS activity, and ameliorates PH. It also prevents short term acclimation to hypoxia via actions on both microglia and astrocytes (118).

However, there are many poorly understood processes in this disease. For example, it is unknown whether the microglia contributing to PH are the resident microglia, bone marrow-derived-microglia-like cells or both. The timing of responses suggests that at least the early events after exposure to PH-inducing stimuli are likely mediated by resident microglia. However, there is a large increase in the number of microglia during later stages of PH progression in animal models of PH. This implies that either bone marrow-derived-microglia-like cells are recruited, or there is a high replication rate of resident microglia, a population of cells previously described to have a low rate of replication until stimulated by brain pathology (35). The phenotypes of the neurons interacting with microglia in the autonomic nuclei in PH are not known. There are multiple neuronal subtypes in autonomic nuclei and many have been shown to respond to short term and intermittent hypoxia (97). Finally, the role of astrocytes in PH have not been explored. Future studies are needed to address these, and other, knowledge gaps.

Taking together the most recent studies and the symptoms commonly observed in pulmonary hypertension, we believe that it strongly argues for a paradigm change in our thinking about pulmonary hypertension. Considering this disease as a syndrome would aid in the development of new targeted medication. It would mean that not only should new medications target the lungs, but also the newly discovered organs/targets that contribute to pulmonary hypertension pathophysiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

5-HT	serotonin
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ACE	angiotensin converting enzyme
ACE2KI	angiotensin converting enzyme 2 knock-in
Ang II	angiotensin II
AT1R	angiotensin II receptor type I
ATP	adenosine triphosphate
BMPR2	bone morphogenic protein receptor 2
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
CX3CL1	Fractalkine
CX3CR1	CX3C chemokine receptor 1
F/B ratio	Firmicutes/Bacteroidetes ratio
FMT	fecal matter transplantation
HMGB 1	high mobility group box 1
ICV	intracerebroventricular
iFABP	Intestinal fatty-acid binding protein
IL	interleukin
IVLM	intermediate ventrolateral medulla
LF/HF	low frequency/high frequency
LPS	Lipopolysaccharides
MCP-1	monocyte chemotactic protein 1
MCT	monocrotaline
MSNA	nerve trafficking activity in muscle sympathetic nerve
NTS	nucleus tractus solitarius
PAH	pulmonary arterial hypertension
PH	pulmonary hypertension
PRR	pattern recognition receptor
PVN	paraventricular nucleus of hypothalamus
RVEF	right ventricular ejection fraction
RVF	right ventricular failure

RVH	right ventricular hypertrophy
RVLM	rostromedullary lateral medulla
RVSP	right ventricular systolic pressure
SNS	sympathetic nervous system
TIMP-1	tissue inhibitor of metalloproteinase 1
TLR4	toll-like receptor 4
TNF	tumor necrosis factor
VEGF	vascular endothelium growth factor
WHO	World Health Organization

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Figure 1.

A: Time-lapse phase contrast microscopic images depicting movement of microglia towards neuron resulting in intimate neuron-microglia interaction. Brain cells in primary culture from hypothalamic-brain stem region were established as described (57). Phase contrast microscopy was used to observe movement of microglia and time-lapse images were recorded (1 frame/15 sec over 1 hour). B: A representative image showing interactions between microglia and neurons in fixed brain section. PVN section was subjected to immunocytochemistry with the use of microglia selective (Iba1+, green) and neuron selective (NeuN, red) antibodies as established previously (104, 105, 112).

Figure 2.

The classical view of PH is shown on the left-hand side of the figure in gold. This view of PH describes changes in the pulmonary vasculature that induce increased afterload to the heart leading to right ventricular failure, and is the target of current medical therapy, shown in blue. The novel view of PH is presented in beige on the right-hand side of the figure. It demonstrates the complexity of this disease from a systemic point of view, considering PH as a sequence of events involving brain, bone marrow cells, gut and its microbiota. The microglial activation and neuroinflammation in autonomic areas may be the starting point for autonomic imbalance, and key to inducing lung inflammation, right ventricular hypertrophy and consequently failure, gut inflammation, altered gut microbial composition and increased bone marrow cell recruitment (green). These changes interact and reinforce one another to contribute to disease progression.

The known actions (+) and possible targets (?) of emerging therapies such as the blockade of serotonin (5-HT, red) and angiotensin II (AngII, purple) are also shown. *Abbreviations:* PAP, pulmonary arterial pressure; RVSP, right ventricular systolic pressure; PDE, phosphodiesterase; NO-sGC, nitric oxide-soluble guanylyl cyclase; SNS, sympathetic nervous system; PNS, parasympathetic nervous system.