Pulmonary arterial hypertension: a disease of tethers, SNAREs and SNAPs?

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Sehgal PB, Mukhopadhyay S. Pulmonary arterial hypertension: a disease of tethers, SNAREs and SNAPs? Am J Physiol Heart Circ Physiol 293: H77–H85, 2007. First published April 6, 2007; doi:10.1152/ajpheart.01386.2006.—Histological and electron microscopic studies over the past four decades have highlighted “plump,” “enlarged” endothelial, smooth muscle, and fibroblastic cellular elements with increased endoplasmatic reticulum, Golgi stacks, and vacuolation in pulmonary arterial lesions in human and in experimental (hypoxia and monocrotaline) pulmonary arterial hypertension. However, the contribution of disrupted intracellular membrane trafficking in the pathobiology of this disease has received insufficient attention. Recent studies suggest a pathogenetic role of the disruption of intracellular trafficking of vesicular proteins and cell-surface receptors in the development of this disease. The purpose of this essay is to highlight the molecular regulation of vesicular trafficking by membrane tethers, SNAREs and SNAPs, and to suggest how their dysfunction, directly and/or indirectly, might contribute to development of pulmonary arterial hypertension in experimental models and in humans, including that due to mutations in bone morphogenetic receptor type 2.

intraglacial vesicular trafficking; Golgi blockade; N-ethylmaleimidesensitive factor; tethers; nitric oxide; endothelial nitric oxide synthase; caveolin-1; Nef protein

THE PATHOBIOLOGY AND MANAGEMENT of pulmonary arterial hypertension (PAH) are receiving focused attention from the National Heart, Lung and Blood Institute and the United States Congress.1 It is estimated that there are ~15,000 deaths annually in the United States alone from this disease. The hallmark plexiform or oniokinesis lesions in the intra-alveolar pulmonary arteries consist of disordered collections of enlarged (“megalocytic”) endothelial cells, fibroblasts, and smooth muscle cell elements, which lead to reduced arterial lumen, cycles of thrombosis and recanalization, and eventually to progressive right ventricular hypertrophy and cardiac failure (35, 89, 92, 102). Although there is ongoing debate about the contribution of vascular remodeling compared with vasoconstriction in this disease (921), there is a growing consensus that the pulmonary arterial wall in PAH has reduced levels of the vasodilator nitric oxide (NO) (24, 55, 67, 83). Therapeutic regimens in use today aim at vasodilation (epoprostenol, sildenafil, endothelin-1-receptor blockers) (55, 68). Novel, yet to be fully tested therapeutic modalities include the statins (68), serotonin-receptor blockers (32), and various tyrosine kinase inhibitors (57, 84); the eventual alternative is lung transplant (55). The challenge here is to understand the underlying pathobiology of PAH at the subcellular and molecular levels so as to then address therapeutics in a meaningfully targeted manner. Compounding this challenge is the realization that the initiating event(s) in human PAH long precedes the clinical diagnosis (75, 102). Thus experimental models [chronic hypoxia or monocrotaline (MCT) administration to name only two] have been used extensively to investigate these initiating mechanisms and to carry out preclinical evaluation of novel therapeutic approaches (16, 52, 63–65, 85, 88; reviewed in Ref. 75). From the clinical and preclinical studies, the pulmonary arterial endothelial cell (PAEC) has come to be viewed as a major cell type whose dysfunction (enlargement and proliferation) appears to initiate PAH (16, 100, 102).

In humans, in addition to idiopathic PAH of either familial or sporadic etiologies, factors include autoimmune diseases such as scleroderma, viral infections such as human immunodeficiency virus (HIV), and intake of anorexogenic drugs (55, 68). The genetic basis for approximately one-half of the familial cases and one-quarter of the sporadic cases includes autosomal dominant mutations in the cell-surface localized bone morphogenetic receptor type 2 (BMPR2) [BMP cytokines are members of the transforming growth factor-β (TGF-β) family of cytokines, which signal to the cell interior by activation of Smad-family transcription factors] (18, 46). However, the rather low penetrance of these mutations (10–20%) has led to searches for “second-hit” or “modifier” genes (49, 81, 106). The etiology of familial or sporadic PAH cases with wild-type BMPR2 alleles remains under investigation. Indeed, mutations in another TGF-β-associated signaling protein, the activin-like kinase 1, is associated with the development of PAH in patients with hereditary hemorrhagic telangiectasia (29). Although it is known that at least some of the disease-causing BMPR2 mutants can be trapped in the Golgi (80), the issue of a dominant-negative disruption by disease-causing BMPR2 mutants of trafficking of other cargo through the Golgi has not been considered.

This lack of discussion of membrane trafficking in the current PAH literature is all the more remarkable in that there is extensive preexisting electron microscopy data in the older PAH literature over the past four decades showing the presence of “plump” or “enlarged” cellular elements with increased endoplasmatic reticulum-Golgi, vacuolation, and Weibel-Palade bodies (these are exocytic vesicles) in pulmonary arterial lesions in human idiopathic PAH and experimental models of this disease (hypoxia or MCT induced) (31, 38, 43, 58, 59, 89, 90) (see discussion in Ref. 65 for detailed description). It is a purpose of this essay to discuss disruption of membrane trafficking as an underlying contributor to the pathobiology of PAH.


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INTRACELLULAR MEMBRANE TRAFFICKING

Intracellular membrane-fusion events between a specific vesicle and its target membrane are mediated by a group of proteins, including membrane tethers, SNAREs and SNAPs, and the ATPase N-ethylmaleimide-sensitive factor (NSF) (the acronym SNARE stands for a “SNAP receptor” and SNAP stands for “soluble NSF association protein”) (6, 39, 93, 95, 105). Tethers are organelle-specific membrane-associated proteins that interact with specific vesicles at distances of up to 50 nm (39) and serve to bring them close to their target membrane (for example, the Golgi organelle contains at least 12 tethers of the coiled-coil nature and an additional five multisubunit tethering complexes; Ref. 95). Tethers are believed to be specifically recruited to distinct subcellular membranes by the active GTP-bound form of the small GTPases of the Rab, Arf, or Arl (Arf-like) families (95). Once recruited, tethers associated with a target membrane interact with corresponding tethers on an approaching vesicle, and this interaction brings the respective membranes close together (88). Although the specificity of this long-distance tethering is determined by this combinatorial interaction, tethers do not directly mediate lipid bilayer fusion (95).

The short-range interaction at distances up to 25 nm (39) between respective membranes about to undergo fusion and the lipid bilayer fusion event itself is mediated by SNARE proteins through the formation of a four α-helix fusion complex (6, 39, 93, 95). There are at least 38 distinct membrane-associated SNAREs in mammalian cells (93), most of which, but not all, are integral transmembrane proteins. Each cell type expresses a different combinatorial basket of SNAREs on distinct subcellular membranes (6, 39, 93). SNAREs have been variously classified as v-SNAREs (when present on a vesicle membrane) and t-SNAREs (when present on a target membrane) or more recently as R-SNAREs (usually corresponding to v-SNAREs) and Q-SNAREs (usually corresponding to t-SNAREs), based on the central functional amino acid residue in their respective “SNARE” motifs (6, 39, 93). (Confusingly, there are certain SNARE proteins nominally called SNAP23, SNAP25, etc., where the SNAP acronym is written with a suffix numeral reflecting their molecular mass and stands for “synaptosome-associated membrane protein.” These are unrelated to the soluble cytosolic α, β, or γ-SNAP proteins, with the latter SNAP acronym standing for “soluble NSF association protein” written with a Greek letter prefix (6, 33).)

It is presently believed that subsequent to the interactions between membrane tethers, which bring the vesicle and target membrane close together (6, 39, 93), there is formation of a four-chain SNARE complex consisting of one α-helix from the v-SNARE on the vesicle and three α-helices from two or three different t-SNAREs on the target membrane. This is called a trans-SNARE complex, and it provides the second level of specificity for membrane fusion reactions; specific v-SNAREs will only interact with specific sets of t-SNAREs (6, 39, 93).

As examples of the specificity and directional of SNARE function in endothelial cells, Malik and colleagues (73) have delineated the role of the SNARE syntaxin-4 in the trafficking of caveolin-1 (cav-1) from the plasma membrane to the cell interior, whereas Pagano and colleagues (14) have delineated the role of the SNARE syntaxin-6 in the trafficking of cav-1 from the Golgi to the plasma membrane.

“Zippering” of α-helices in a trans-SNARE complex from the free cytosolic ends toward their membrane-anchored domains drives physical lipid bilayer fusion (6, 33, 39, 93). After membrane fusion, the respective v- and t-SNAREs are in a complex on the same membrane (6, 33, 39, 93). This is the cis-SNARE complex. For continuation of repeated rounds of membrane fusion reactions, the cis-SNARE complex has to be disassembled, with separation of the v- and t-SNAREs from each other and transport of the v-SNAREs back to the vesicle/compartment of origin.

Disassembly of all cis-SNARE complexes is mediated by the ATPase NSF. However, NSF is a cytosolic protein and does not directly interact with lipid membranes. Recruitment of NSF to membrane-bound cis-SNARE complexes is mediated by α-SNAP, which is also a soluble cytosolic protein but can directly interact with cis-SNARE complexes (6, 39, 93). Although there are three forms of soluble SNAPs in mammalian cells (α, β, and γ), SNARE disassembly is primarily mediated by the ubiquituous α-SNAP (β-SNAP is neuronal, and the protein called γ-SNAP does not bind SNARE complexes) (33, 96). The recruited NSF then uses its ATPase activity to “pry” the v- and t-SNAREs apart (6, 33, 39, 93).

From the standpoint of the present perspective, it is important to note that NSF is a redox-sensitive protein with nine cysteine residues (54). Cysteine residues of NSF can be covalently modified by NO-mediated S-nitrosylation. This inhibits the ability of NSF to disassemble SNARE complexes without affecting its ATPase activity (54). Mutations in specific Cys residues block the ability of NSF to either associate with the SNARE proteins (mutations on Cys residues 11, 21, 334, 568, and 582) or disassemble them (mutations on Cys residues 91 and 264) (54). NO-mediated nitrosylation specifically affects Cys91 and Cys264, the same residues that have been implicated in mediating SNARE disassembly (54). Functionally, S-nitrosylation of NSF blocks Weibel-Palade body exocytosis in human aortic endothelial cells (54) and the anterograde trafficking of cargo proteins like the vesicular stomatitis virus G protein from the Golgi to the plasma membrane in COS-7 cells (37). Thus the biological function of NSF is highly dependent on its cysteine residues and can be modified by NO-mediated S-nitrosylation.

The activity of various SNARE proteins is regulated by phosphorylation in an on-or-off manner differently for different SNAREs and by association with regulatory proteins such as with members of the Sec/Munc family of proteins (6, 33, 39, 93). Indeed mutations in specific SNAREs and their regulatory Sec/Munc proteins lead to well-defined human diseases such as Parkinson’s disease (α-synuclein dysfunction) (17), amyotrophic-renal dysfunction-cholestasis syndrome (VPS33B dysfunction) (25), and familial hemophagocytic lymphohistiocytosis (Munc 13-4 dysfunction) (61) to name only a few (also see Refs. 33 and 93 and citations therein). The proteolytic cleavage of SNAP25 by botulinum toxin and of vesicle-associated membrane protein 1 (VAMP1), VAMP2, and VAMP3 species by tetanus toxin underlies the irreversible inhibition of neuronal function by these toxins (reviewed in Ref. 93). Moreover, the Golgi tether giantin is known to be a major autoantigen in several autoimmune diseases (70, 82, 98).

In this context, it is intriguing that anorexogenic drugs, as a broad-brush concept, are known to affect membrane receptor trafficking in the nervous system (21).
GOLGI BLOCKADE IN PAH

In investigating the initiating mechanism leading to the development of PAH in the MCT-rat model, we observed a loss of cav-1, the structural component of caveolae and cell-surface rafts, from PAEC in vivo (52). [In vivo, the MCT is converted to the bioactive pyrrolic derivative MCTP in the liver, and it is this pyrrolic derivative that affects PAEC in lungs to trigger a cascade of changes culminating in PAH 10–14 days later. Thus cell culture experiments in endothelial and epithelial cells require the use of MCTP per se.] This loss of cav-1 was accompanied by the reciprocal hyperactivation of promitogenic Tyr-P-STAT3 (PY-STAT3) and ERK1/2 signaling and DNA synthesis (52) (Fig. 1). These observations have been recently confirmed by Jasmin et al. (40) who went on to show that amelioration of MCT-induced PAH by the cav-1 scaffolding domain peptide (AP-cav-1) inhibited the loss of cav-1 and prevented hyperactivation of PY-STAT3. The significance of these observations in the pathobiology of PAH is emphasized by the recent reports of a loss of cav-1 in endothelial cells in the plexiform lesions in idiopathic PAH in humans and in a hypoxia-based PAH model in the mouse (1, 97). Moreover, the cav-1−/− knockout mouse also develops PAH spontaneously (108).

Mechanistically, the loss of cav-1 from cell surface rafts/caveolae in PAEC was accounted for by the trapping of cav-1 in the Golgi (52, 85, 88) (Fig. 1). In cell culture, MCTP- or hypoxia-induced endothelial cell megalocytosis was characterized by marked enlargement of the Golgi organelle into a circumnuclear structure, as assayed by immunofluorescence of the Golgi tether GM130 (52, 63–65, 85, 88). A block of anterograde trafficking through the Golgi after MCTP was also confirmed using an assay for secretion of horseradish peroxidase (85). In addition to the trafficking block, MCTP also disrupted the mitosis-sensor and apoptosis-sensor functions of the Golgi in that these cells failed to enter mitosis and remained alive for an extended time (63, 88). Thus these megalocytotic cells became hyperploid, enlarged, and vacuolated with a "growing but nondividing" phenotype (2, 88).

The block in cell-cycle traverse was novel in that MCTP-treated endothelial and epithelial cells showed a decrease in total cellular levels of cdc2 kinase, the cyclin-dependent kinase essential to initiate mitosis (63), although there was increased Ser phosphorylation of the Golgi mitosis sensor GM130 (a cdc2 substrate whose phosphorylation is typically a prelude to Golgi fragmentation and entry into mitosis) (88). Despite increased Ser phosphorylation of GM130 in such cells, there was no Golgi fragmentation or entry into mitosis (63, 88). Moreover, the increased accumulation of the Golgi tether and scaffolding protein p115 in its intact form (see below), which is also an apoptosis sensor in that the proteolytically cleaved 30-kDa fragment of p115 enhances apoptosis, suggested a possible mechanism for why these cells might not undergo apoptosis (12, 65, 85).

Thus “the Golgi blockade hypothesis” refers to both a disruption of membrane trafficking and of cell cycle traverse, resulting in growing but nondividing cells. That a similar phenotype was observed after hypoxia (65) increased the physiological relevance of these observations.

SNAREING PULMONARY HYPERTENSION

In trying to understand the underlying subcellular mechanisms for the anterograde trafficking block through the Golgi, cell fractionation and immunofluorescence techniques revealed the marked trapping not only of cav-1 and endothelial nitric oxide synthase (eNOS) in the Golgi but also of BMPR2 and of diverse Golgi tethers, SNAREs and SNAPs (GM130, p115, giantin, golgin 84, clathrin heavy chain, syntaxin-4, syntaxin-6, Vti1a, Vti1b, GS15, GS27, GS28, SNAP23, and α-SNAP) in the enlarged, circumnuclear Golgi organelle in megalocytotic endothelial and epithelial cells (65, 85) (Fig. 1). That diverse

Fig. 1. SNAREing pulmonary hypertension. BMPR2, bone morphogenetic receptor type 2; cav-1, caveolin-1; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; NSF, N-ethylmaleimide-sensitive factor; SNAP, soluble NSF association protein; SNARE, SNAP receptor. (Modified from Refs. 65 and 85.)
tethers, SNAREs and SNAPs, were trapped in the Golgi suggested interference with the disassembly step in membrane trafficking. Indeed, NSF, the ATPase required for disassembly of all cis-SNARE complexes, was found to be largely sequestered in an intracellular location separate from the Golgi (85). This would explain the accumulation of diverse tethers, SNAREs and SNAPs, in the Golgi due to inhibition of SNARE complex disassembly after MCT. Exposure to hypoxia in cell culture also produced enlarged PAECs with enlarged Golgi and the accumulation of tethers, SNAREs and SNAPs (65). Immunofluorescence studies of lung tissue from MCT-treated rats confirmed enlargement of the perinuclear Golgi in lung arterial endothelial and parenchymal cells as early as 4 days after MCT administration, namely, at a time that preceded the appearance of PAH (which usually develops by 10–14 days) (85). We thus suggested that PAH, at least that after MCT or hypoxia, might be looked upon as a disease of dysfunctional Golgi tethers, SNAREs and SNAPs with the redox-sensitive NSF being a key molecular target (65, 85) (Fig. 1).

PAH DUE TO OVEREXPRESSION OF THE MEMBRANE-TRAFFICKING REGULATOR PROTEIN Nef

In an insightful study, Marecki et al. (51) investigated the development of PAH in a macaque model. In this primate model, animals infected with simian HIV develop PAH. Working with two virus strains, these investigators observed that macaques infected with simian HIV expressing the protein Nef, but not virus deleted in Nef, developed PAH with Nef expression in endothelial cells in the pulmonary arterial lesions. These investigators also showed that human arterial lesions in HIV-related PAH contained endothelial and certain factor VIII-negative cellular elements (probably vascular smooth muscle and epithelial and mononuclear elements) overexpressing the Nef protein (51).

Extensive prior work from several cell biology laboratories has identified Nef as a protein that regulates outward trans-Golgi and also inward endocytic trafficking (8, 11, 50, 78, and citations therein). A specific block is in the trans-Golgi to plasma membrane trafficking (a Golgi blockade) (78). Nef also disturbs the morphology of the early/recycling endosome compartment and reduces the rate of transferrin receptor recycling, causing its accumulation in the endosomal compartment (50). Furthermore, Nef colocalizes with clathrin-coated pits and vesicles and can directly interact with the adaptin protein 2 complex, leading to increased clathrin-mediated endocytosis with subsequent downregulation of the CD4 protein (8, 11). Moreover, additional studies have shown that increased expression of Nef traps a host of cellular proteins, including the major histocompatibility class I molecules in the Golgi compartment (Ref. 78 and citations therein). However, the consequences of Nef overexpression on Golgi tethers, SNAREs and SNAPs, are not known.

Thus, at the very least, there is now genetic evidence in a primate model of a potential causal relationship between a protein that disrupts intracellular membrane trafficking and the development of PAH.

CONSEQUENCES OF A TRAFFICKING BLOCK: REDUCED CAVEOLAR NO WITH INTRACELLULAR SEQUESTRATION OF eNOS

There is a growing consensus that there are reduced NO levels in the pulmonary arterial walls in human and experimental PAH, even though the levels of eNOS protein have been variably reported as unchanged, decreased, or even increased (24, 34, 40, 41, 53, 67, 76, 83, 101). It is also now clear that the extracellular NO derives from cell-surface caveolar eNOS and that intracellularly active eNOS produces NO in subcellular compartments from where the NO does not reach the extracellular space (27, 37).

Murata et al. (66) had previously shown that eNOS was cotrapped in the Golgi with cav-1 in endothelial cells exposed to hypoxia. Our group (65) recently observed that eNOS in endothelial cells exposed to MCTP or hypoxia or after senescence was increasingly sequestered in the Golgi and in a non-Golgi cytoplasmic vesicular compartment partially overlapping with the endoplasmic reticulum (65). Under these conditions, there was a loss of cell surface caveolar eNOS with colocalization between some of the cell-centric eNOS with Golgi tethers and SNAREs (65). Live-cell imaging studies of NO using DAF-2DA as a membrane-permeant subcellular reporter revealed a loss of caveolar NO with marked increase in cell-centric accumulations of NO (65). The aberrant sequestration of eNOS in an intracellular compartment away from cell-surface caveolae would account for the reduced NO in the pulmonary arterial vasculature despite sustained or even increased protein levels of eNOS (Fig. 1).

The sequestration of eNOS in a cell-centric compartment in endothelial cells after MCTP, hypoxia, or senescence from within which the enzyme continues to generate NO but which does not reach the exterior of the cell might well initiate a self-amplifying loop leading to increased S-nitrosylation of NSF and a further inhibition of trafficking (Fig. 1). In fact, Sessa et al. (37) reported very recently that Golgi-targeted mutants of eNOS produced NO locally within this subcellular compartment and thus modified intracellular NSF by S-nitrosylation. The consequence was a block in trafficking through the Golgi of cargo proteins such as the vesicular stomatitis virus G protein (37). Thus, from the point of view of this essay, we suggest that the aberrantly sequestered eNOS after MCTP, hypoxia, or senescence would generate intracellular NO, which would further inhibit NSF and membrane trafficking through the Golgi in a self-amplifying inhibitory loop (Fig. 1). However, this eNOS/NO/NSF inhibitory loop can represent only one aspect of the PAH disease mechanism in that eNOS<sup>-/-</sup> knockout mice have enhanced susceptibility to hypoxia-induced PAH (23, 74).

CONSEQUENCES OF A TRAFFICKING BLOCK: MITOCHONDRIAL DYSGENESIS?

Archer and colleagues have recently reported that endothelial cells derived from human PAH lesions and the lesions themselves as well as those in fawn-hooded rats, which spontaneously develop PAH, showed dysmorphic mitochondria, the loss of superoxide dismutase 2 from such mitochondria, and downregulation of the Kv1.5 potassium channel (7, 60). Thus they suggested that PAH might be a mitochondrial disease. The downregulation of Kv1.5 would inhibit apoptosis and cause an
increase in calcium-induced vasoconstriction. The Kv1.5 potassium channel protein, which interacts directly with cav-1 and with the serotonin receptor in communoprecipitation assays (15), traffics to the cell surface through the Golgi organelle (13). This trafficking requires the dynein motor complex (which mediates anterograde vesicle transport) and, importantly, is enhanced by the Golgi tether Golgin-160 (13). Thus a block in trafficking through the Golgi to the cell surface, as suggested by us, could also lead to downregulation of the Kv1.5 channel at the plasma membrane and consequent mitochondrial dysfunction as suggested by Archer and colleagues (7, 60). It would be intriguing to investigate markers of anterograde trafficking through the Golgi in endothelial cell lines derived from idiopathic PAH patients and to investigate whether mitochondrial proteins and the Kv1.5 channel protein are trapped in the Golgi in such cells.

EFFECTS OF PAH-CAUSING BMPR2 MUTANTS ON MEMBRANE TRAFFICKING?

To what extent might BMPR2 mutations found in familial and sporadic PAH initiate the disease process through dysfunction of intracellular trafficking? To put this question into perspective, we note that it is known from the breast cancer literature that the trapping of mutant cav-1 species in the Golgi (such as of the P132L mutant) results in a dominant-negative block of anterograde trafficking of wild-type cav-1 (47). Furthermore, it is known from the literature relating to autosomal-dominant polycystic disease of the kidney that disease-causing mutations in the cell-surface protein polycystin-1 result in trapping of the mutant protein in the Golgi, leading to a dominant-negative block of trafficking of other wild-type cellular proteins such as E-cadherin and cav-1 (9, 10). As with BMPR2 mutation-associated PAH, which is also autosomal dominant but with low penetrance, a discussion regarding “second hits” and late penetrance (the kidney disease can appear as late as the eight or ninth decade) is taking place in the autosomal-dominant polycystic disease of the kidney field (44, 72).

 Trafficking from the cell interior to the plasma membrane. Heterogenous mutations in the extracellular ligand binding, kinase and the cytoplasmic tail domains of the BMPR2 receptor gene have been associated with the development of PAH (18, 46, 69, 80). It has been demonstrated that cysteine substitution mutations in the extracellular ligand binding domain (like C60Y) block the trafficking of the mutant BMP2 receptor protein to the plasma membrane with increased retention and sequesterion in intracytoplasmic compartments (69, 80). Furthermore, cysteine substitution mutants in the kinase domain (like C347Y) are also largely sequestered in intracytoplasmic, perinuclear compartments (69, 80). Although the noncysteine mutants in the kinase and cytoplasmic tail domains have been reported to traffic correctly to the cell surface, inspection of the published data reveals that, in fact, there is significant juxtanuclear and cytoplasmic punctate sequestration of these BMP2 mutants compared with that for wild-type BMP2 (in Ref. 69, see Fig. 5 and compare panels A, D, and E). The juxtanuclear sequestration of these mutant BMP2 proteins is clearly indicative, on its face, of dysfunction of intracellular trafficking. Moreover, it has been shown that mutant BMP2 species can bind to wild-type BMPR1, exerting a dominant-negative effect on the trafficking and function of wild-type BMPR1 (69). Furthermore, although some of the noncysteine mutants in the kinase and tail domains of BMP2 may reach the plasma membrane and activate Smad signaling (69, 80), their partial intracytoplasmic retention may be sufficient to initiate a dominant-negative effect on trafficking.

 Trafficking from the plasma membrane to the cell interior. In this discussion, it is pertinent to emphasize that recent data in the cell biology literature show that transcytoplastic transit of transcriptionally productive signaling from the plasma membrane to the cell interior and the nucleus by signaling pathways involving the Smad family (20, 36, 71, 99) and STAT3 (87) transcription factors is associated with endocytic/caveolar vesicular trafficking, which, by definition, must thus involve NSF, vesicle tethers, SNAREs, and SNAPs (Fig. 1). For instance, it has been shown that transcriptionally productive TGF-β signaling involving Smad transcription factors takes place along the early endocytic pathway (20, 36, 71, 99) and that the Smad proteins are recruited to the respective activated TGF-β-receptor in the early endosome membrane by the adapter protein called “Smad anchor for receptor activation” (SARA) (36, 99). The role of SARA is to “present” specific R-Smads for phosphorylation by the activated TGF-β receptor (36, 99). Moreover, transcriptionally productive BMP2/Smad1,5 signaling has been shown to be largely dependent on the integrity of the clathrin-mediated endocytic pathway (30).

Nevertheless, the present discussion in the PAH field about BMP/Smad-signaling lacks the concepts of membrane-associated trafficking in carrying a productive signal from the plasma membrane to the cell interior (as one example, see Fig. 1 of Ref. 62, which specifically attempts to depict Smad signaling in PAH in the BMP2 context with the space between the plasma membrane and the nucleus depicted as devoid of any membrane structures). A dysfunction of NSF, tethers, SNAREs, and SNAPs would also affect this inward signaling pathway (Fig. 1).

The considerable heterogeneity of disease-causing BMPR2 mutations (18, 46, 69), the limited ability of haploinsufficiency of BMP2 to cause PAH without further environmental stress factors (3, 48, 91, 103), and inability of gene-transferred wild-type BMP2 to always reverse established PAH (56, 77) suggest that, although mutations in BMP2 may predispose to the development of PAH, it is unlikely that these are solely responsible for the development of the disease. Given that TGF-β/Smad (20, 36, 71, 99) and BMP/Smad (30) signaling are membrane associated, the role of membrane trafficking and thus of vesicle tethers, SNAREs, and SNAPs needs to be included in mechanistic discussions of inward signaling events involved in PAH.

IL-6 IN PAH

Relevant to mechanistic considerations leading to the hyperproliferative disordered state of endothelial, smooth muscle, and fibroblastic elements in plexiform lesions in idiopathic PAH (sometimes called a “pseudomalignant” state), we note that 1) IL-6 is one of the cytokines invariably found in and around PAH lesions (4) (reviewed in Refs. 52, 28), 2) IL-6 has previously been identified as an invariant presence in the host-tumor interaction and is a “scattering factor” for epithelial and endothelial cells (79, 86), 3) IL-6 has been shown to
enhance vascular smooth muscle cell proliferation (104), 4) transgenic mice overexpressing IL-6 in the lung develop mild PAH and a more severe PAH in response to hypoxia than nontransgenic littersmates (26), 5) IL-6/gp130 signaling is upregulated by PAH-related BMPR2 mutant in transgenic mice (28), and 6) IL-6 enhances BMP-responsive transcriptional signaling in smooth muscle cells in culture and, more generally, IL-6/gp130 and BMP/BMPR2 signaling pathways cross-modulate each other (28). Together, these data point to IL-6 as a key causal contributor to the pathobiology of PAH. That both IL-6 and BMP-signaling from the plasma membrane to the cell interior transit along the vesicular endocytic and caveolar pathways provide a physical platform for these cross-regulatory interactions and their modulation by tethers, SNAREs and SNAPs (Fig. 1 and Refs. 30 and 87).

DISCORDANT REPORTS CONCERNING THE Ang1/Tie2 PATHWAY IN PAH

Stewart and colleagues (45, 107) have pointed to a loss of the Tie2 receptor for the growth factor angiopoietin-1 (Ang1) from endothelial cell elements in pulmonary arteries after MCT or hypoxia in rats and the ability of cell-based gene-transferred Ang1 to ameliorate PAH due to MCT or hypoxia. This cell-based Ang1-gene transfer by itself did not produce PAH (107). In contrast, Thistlethwaite and colleagues (94) used an adenovirus vector-based gene transfer approach to introduce Ang1 into rats and report that this by itself produced PAH (94). In the hands of the latter investigators, gene-transfer-based expression of soluble Tie2 fragment, an inhibitor of Tie2 function, ameliorated MCT- and Ang1- but not hypoxia-induced PAH in rats (42). Furthermore, although Thistlethwaite and colleagues report an increase in Tie2 phosphorylation with no change in total Tie2 protein levels in extracts of lungs of rats with pulmonary hypertension after Ang1-gene transfer (94) or of patients with idiopathic PAH and PAH associated with other diseases (22), Eddahibi and colleagues (19) report a fourfold increase in total Tie2 levels in lungs of patients with idiopathic PAH and a parallel increase in phosphorylated Tie2. In contrast Stewart and colleagues (45) report no change in phosphorylated Tie2 in lungs of patients with idiopathic PAH or PAH associated with other diseases. Finally, although Thistlethwaite and colleagues (22) report increased Ang1 in lungs of patients with PAH, Stewart and colleagues (45) and Eddahibi and colleagues (19) failed to observe any changes in Ang1 or Ang2.

What is remarkable in the above-cited discordant literature is that the investigators, although cognizant of their respective discordant data, pay scant attention to the mechanisms of intracellular trafficking of components of the Ang1/Tie2 signaling pathway and consequences of disruption of this trafficking differently in different experimental models and in different patients (Fig. 1). As with the BMPR2 receptor and other vasorelevant cell-surface proteins, anterograde trafficking of the Tie2 receptor and secretion of the Ang1 cytokine itself would require Golgi to plasma membrane (outward) vesicular transit. Moreover, it is well established that Ang1 causes rapid internalization of 70% of the plasma membrane pool of phosphorylated Tie2 receptor within 30 min in endothelial cells followed by degradation of Tie2 (5). We suggest that it is necessary to include considerations of disrupted intracellular trafficking (Fig. 1) in discussions of the changes in the Ang1/Tie2 pathway in the pathobiology of PAH.

CONCLUDING COMMENTS

The major objective of this essay was to bring aspects of intracellular membrane trafficking and its vocabulary into discussions of the pathobiology of PAH. Together, the available data clearly point to disruption of membrane trafficking within cellular elements that make up the pulmonary arterial lesions in humans and in experimental models of PAH. Existing puzzles relating to the mechanisms by which mutations in BMPR2 cause an autosomal dominant disease with low penetrance might be amenable to resolution in light of the additional entanglement of regulatory molecules involved in membrane trafficking, the tethers SNAREs and SNAPs. Rapid discovery screens to find small molecules that “unblock the Golgi block” might yield novel therapeutic solutions for the management of PAH in humans.

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