Diverse Pharmacology of Prostacyclin Mimetics: Implications for Pulmonary Hypertension

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Abstract

Pulmonary arterial hypertension (PAH) is a progressive vascular remodelling disease where patients ultimately die from heart failure. Increased production of vasoconstrictors (endothelin-1 and thromboxane A_2) accompanied by loss of prostacyclin, nitric oxide (NO), bone morphogenetic protein receptor type 2 (BMPR2) and TASK-1 combine to cause endothelial apoptosis, smooth muscle hyperactivity and thickening of the blood vessel wall. Prostacyclin remains the most efficacious treatment for PAH, and several prostacyclin analogues are approved for use via different administration routes. They act as vasodilators but potently inhibit platelet aggregation, cell proliferation and inflammation. The pharmacology of each prostacyclin (IP) receptor agonist is distinct, with other targets contributing to their therapeutic and side-effect profile, including prostanoid EP_1, EP_3, EP_2 and DP_1 receptors, alongside peroxisome proliferator-activated receptors (PPARs), to which prostacyclin and some analogues directly bind. To improve selectivity, selexipag, a non-prostanoid was developed, whose only significant biological target is the IP receptor, but is a partial agonist in cyclic AMP assays and has no anti-aggregatory properties in vivo. Prostanoid receptor expression profiles in the normal and diseased lung demonstrate loss of the IP receptor and upregulation of EP_2 and EP_3 receptors in PAH, affecting the action of prostacyclin mimetics in different ways. We discuss how prostacyclins

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might rescue BMPR2 and TASK-1 dysfunction and the importance of EP_2 receptors as negative modulators of vascular tone, proliferation and fibrosis. Alongside DP_1 and EP_4 receptors, they have specific roles in veins and airways. Whether drugs selective for the IP receptor confer a superior or reduced therapeutic benefit remains an important clinical question as do the role of platelets in PAH.

Keywords
Prostanoid receptors · Prostacyclin mimetics · Vascular smooth muscle · Pulmonary hypertension · Cell proliferation

5.1 Introduction

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease. Patients suffer from elevated pulmonary artery pressure (PAP) and right ventricular (RV) heart failure [1]. Without treatment, adults have a median life expectancy of 2.8 years from diagnosis, while children have less than 10 months [2, 3]. Narrowing of the small pulmonary arteries involves proliferation of endothelial cells, smooth muscle cells and fibroblasts and is associated with inflammatory cell infiltration and the formation of thrombotic lesions [4, 5]. The highly proliferative phenotype is driven by elevated levels of mitogens (endothelin-1, thromboxane A_2 and 5-HT) and growth factors (platelet-derived growth factor, transforming growth factor β1 and fibroblast growth factor) concomitant with a reduction in the endogenous levels of nitric oxide (NO) and prostacyclin, agents which have potent anti-proliferative and anti-inflammatory properties in many different cell types [6].

Pulmonary hypertension (PH) is classified by the World Health Organization (WHO) into five clinical groups, with patients diagnosed as having PAH belonging to Group I [5, 7]. PAH is further classified as being idiopathic (of no known cause), heritable (genetic component) or secondary to other lung or systemic diseases, including connective tissue disease (CTD), congenital heart disease (CHD) and HIV [5, 7]. It is estimated that around 10% of all patients are described as having heritable PAH (HPAH). From this, a significant proportion of patients (~70–80%) have a mutation in the bone morphogenetic protein receptor type 2 (BMPR2), a member of the transforming growth factor beta (TGF-β) receptor family [7, 8]. The consequence of such mutations is to lead to a pro-proliferative phenotype in pulmonary arterial smooth muscle cells (PASMCs) and a high vascular permeability in endothelial cells leading to apoptosis [9, 10]. It is also important to note that down-regulation of the BMPR2 protein occurs in around 25% of patients with idiopathic PAH (IPAH) [8, 11].

Additional and rarer heterozygous germline mutations in the TGF-β pathway have also been implicated, with studies confirming activin-like kinase-type (ALK-1), endoglin and various members of the SMAD (SMAD 9, 4, 5, 1) family contributing to the pathogenesis of HPAH [8]. ALK is a type-1 receptor phosphorylated when BMPR2 is activated by a BMP ligand and endoglin encodes a type III or

accessory receptor to BMPR2, while phosphorylation of SMAD 1,5 and 8(9) leads to their association with the nuclear chaperone SMAD4, resulting in translocation of the SMAD complex to the cell nucleus to regulate transcription [8]. Furthermore, loss of function mutations in the potassium channel KCNK3 (TASK-1) have been described in patients with either HPAH or IPAH, representing a mechanistically novel cause of PAH [12]. This parallels the downregulation of Kv channels that has been implicated to underlie hypoxic-induced vasoconstriction and abnormal proliferation of smooth muscle cells from patients with IPAH [12].

5.2 Development of Prostacyclin Mimetics and Their Diverse Pharmacology

Prostacyclin, a prostanoid produced in endothelial and smooth muscle cells by the cyclooxygenase (COX) pathway, was discovered in 1976 by a group of scientists led by John Vane as a potent vasodilator and anti-thrombotic agent of the vasculature [13, 14]. It was the first treatment to routinely be used in PAH, with the primary clinical benefit resulting from a reduction in PAP and resistance thereby improving cardiac output [15]. Shortly thereafter, chemically stable analogues of prostacyclin with a longer half-life in the blood were synthesised, including beraprost, iloprost, treprostinil and cicaprost. Apart from cicaprost, these analogues have been in use for PAH since the early to mid-1990s, with iloprost and treprostinil receiving FDA approval in 2002 and 2004, respectively, whereas beraprost is currently only licensed in Asia. Initial work presumed prostacyclin drugs produced their effects via binding to the Gs-coupled prostacyclin (IP) receptor leading to the activation of adenylate cyclase and the cellular accumulation of intracellular cyclic-3′,5′-adenosine monophosphate (cAMP) [16]. Consistent with this notion, the blood pressure–lowering, vasodilatory and anti-aggregatory properties of cicaprost were abolished in IP receptor null mice [17]. Indeed the IP receptor appears to largely account for the pharmacological properties of prostacyclins in humans, including vasodilator [18, 19] and anti-proliferative effects [20–22] in normal pulmonary smooth muscle as well as inhibition of platelet function in healthy individuals [23, 24]. However, early on in their development, prostacyclin and its analogues were found to regulate gene transcription via peroxisome proliferator-activated receptors (PPARs), which would concur with the strong perinuclear expression of prostacyclin synthase [13] with cyclooxygenase enzymes [25], both the enzymes required for prostacyclin synthesis. Some analogues (but not cicaprost) were shown to bind and activate PPARα and PPARβ, an effect reported to be independent of the IP receptor and cAMP generation [26, 27]. Such a mechanism might help to explain the enigmatic observation (at the time) that abolition of the iloprost-induced elevation in cAMP by adenylate cyclase inhibition had no significant effect on vascular relaxation by this agent in guinea-pig aorta [28]. However, it was not until a decade later did evidence emerge for the explicit role of PPARs in mediating some of the functional effects of prostacyclin analogues on vascular tone, cell proliferation and endothelial cell integrity (reviewed in [6]). Indeed, studies in genetically modified mice show that distinct

phenotypes are generated when the IP receptor and the prostacyclin synthase gene are deleted; the former are normotensive but do show an increased susceptibility to thrombosis, while the latter are hypertensive and show fibrosis and vascular remodelling in the kidney [6]. This strongly suggests that PPARs are involved in regulating blood pressure and is consistent with the known vasodilatory effects of ligands activating all three PPAR (α, β, γ) isoforms [29, 30].

At the same time as the discovery of signalling through PPARs, prostacyclin and its analogues were found to activate multiple prostanoid receptors, including EP_1, EP_3, EP_4, TP and FP [16, 31]. Given that all (bar EP_4) are contractile receptors linked to either calcium elevation or inhibition of cAMP generation (Fig. 5.1), and thus their activation might promote unwanted side effects or physiologically oppose IP receptor signalling, prompted the search for more selective IP receptor agonists. Based on this rationale, selexipag, a novel non-prostanoid moiety with a high selectivity for the IP receptor was developed [32, 33] and subsequently approved in 2015 for the treatment of PAH [34]. Selexipag, which is structurally different from prostacyclin, is broken down in the liver to form MRE-269 (known also as ACT 333679), a metabolite with a significantly increased affinity for the IP receptor but with essentially weak or no activity at other prostanoid receptors [32]. Along the same lines, a new series of potent, non-prostanoid and relatively

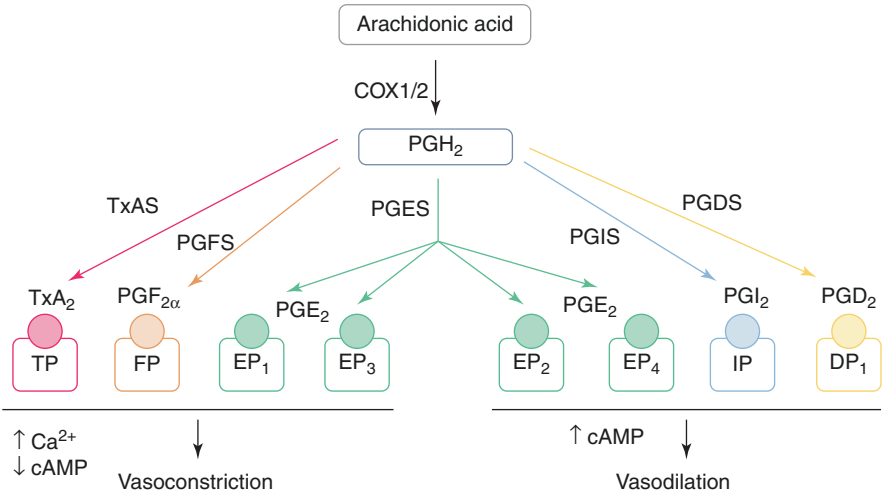

Fig. 5.1 Synthesis of prostanoids and their downstream receptors. Oxidation of arachidonic acid by cyclooxygenase (COX-1 and COX-2) enzymes converts it to an unstable intermediate, prostaglandin H_2, the common precursor for five principal bioactive derivatives. Thromboxane A_2 (TXA_2), prostaglandin $F_{2\alpha}$, ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2), prostacyclin and prostaglandin D_2 (PGD_2) are then generated by the action of individual prostaglandin synthase enzymes (TxAS, PGFS, PGES, PGIS and PGDS). Once generated, the different prostanoids exert their biological effects by binding to and activating specific membrane bound G-protein-coupled receptors. TP, FP, EP_1 and EP_3 receptors are contractile receptors coupled to Gq and Gi that either elevate intracellular $Ca^{2+}$and/or reduce cAMP, respectively. EP_2, EP_4, IP and DP_1 receptors are vasorelaxant receptors coupled to Gs that will activate adenylate cyclase and elevate intracellular cAMP

selective IP receptor agonists were recently synthesised, with ralinepag (APD811) in clinical development as a next generation, once a day oral for the treatment of PAH [35]. Other novel approaches are being taken, including the development of ONO-1301, a non-prostanoid with a dual function of activating the IP receptor and inhibiting TXA_2 synthase [24]. The latter property of the molecule appears to protect the IP receptor from undergoing desensitisation, which is often observed with repeated prostacyclin or analogue administration. This suggests a major role for the TP receptor in promoting IP receptor desensitisation.

All prostacyclin mimetics used clinically display high affinity binding to the human IP receptor. Potency varies, with those having a Ki of 2–4 nM (prostacyclin, iloprost and ralinepag) and those having a tenfold lower affinity with a Ki of 20–38 nM (MRE-269, treprostinil and beraprost), or lower still, with selexipag having a Ki of 250 nM [6, 35]. Esuberaprost (beraprost-314*d*), a reformulated single isomer of beraprost, may be the most potent IP agonist developed so far for therapeutic use [36] and is now in a phase III clinical trial as an oral with inhaled treprostinil. It remains to be determined whether high selectivity towards the IP receptor, which typifies the two non-prostanoid IP receptor agonists, will give rise to therapeutically superior effects and/or a better side-effect profile compared to prostacyclin mimetics that can act at other membrane and nuclear receptors.

Mechanistic and functional differences between prostanoid and non-prostanoid IP agonists are now beginning to emerge that need to be carefully considered in a clinical context. Both ralinepag and MRE-269 behave as partial agonists in cAMP assays in CHO cells stably expressing the human IP receptor and in human PASMCs from PAH patients [35, 37, 38]. Partial agonism may in part explain why MRE-269 was about 65-fold less potent than iloprost at inhibiting contractions in human pulmonary arteries [19] and why selexipag has little overall effect on platelet aggregation in vivo [39]. The latter might suggest the inability of the active metabolite to raise cAMP levels sufficiently to oppose platelet aggregation by endogenous mediators like thromboxane A_2 (TXA_2), which in itself can lower cAMP levels through IP receptor desensitisation, Gi coupling to adenylate cyclase or Ca^{2+} activation of cAMP-dependent phosphodiesterases [6, 24]. High EP_3 receptor activity is also likely to have a similar effect as this receptor is negatively coupled to adenylate cyclase.

The role of platelets as a disease-modifying process in PAH is neither well understood nor the nature of the benefit derived from anti-coagulant therapy [40, 41]. This is an issue that needs to be further investigated as activated platelets produce and release vasoactive substances such as TXA_2, 5-HT and platelet-derived growth factor [41, 42], which may cause harmful vasoconstriction and vascular remodelling in PAH. However, as internal bleeding is a potential risk factor for all prostacyclin analogues [41], and thrombocytopenia a complication of long-term use with epoprostenol (synthetic prostacyclin) in some PAH patients [43], the ability to switch to an IP agonist devoid of anticoagulation properties might be considered beneficial in some patients.

There are also key mechanistic differences in how prostacyclin analogues and non-prostanoid IP agonists inhibit pulmonary arterial smooth muscle proliferation in cells derived from PAH patients. The anti-proliferative responses to treprostinil and iloprost show weak effects of IP receptor inhibition with RO1138452 [22, 44] whereas the same antagonist completely reversed the effects to both MRE-269 and ralinepag, suggesting inhibition of cell proliferation was fully IP receptor-dependent [37, 44]. These results suggest that non-IP receptor targets play a significant role in mediating the effects of some prostacyclin analogues in PAH whereas the IP receptor is likely to be the only significant biological target for MRE-269 and ralinepag. In the search for an explanation to the original 2010 observation, radioligand-binding assays against human prostanoid receptors demonstrated unexpectedly that treprostinil had a tenfold higher affinity for the DP_1 and EP_2 receptor (Ki 4.4 and 3.6 nM, respectively) than the IP receptor [45]. On the other hand, iloprost had a ≥240-fold lower affinity for DP_1 and EP_2 but was equipotent at the IP and EP_1 receptor [45]. A similar potency pattern was observed in functional assays (cAMP and calcium) in the same study, confirming the unique pharmacology of treprostinil [6], which was subsequently independently verified [46]. It is known that receptor affinities can differ significantly between species for natural ligands as well as for their agonists [6] and antagonists [47] so future experiments should be directed towards obtaining the binding affinity profile of treprostinil at mouse and rat prostanoid receptors (and for that matter some prostacyclin drugs) and how different models of PAH affects receptor expression in relevant tissues and cells.

The receptor selectivity of prostacyclin and its mimetics could greatly impact on their control of pulmonary haemodynamics and vascular remodelling in PAH as well influence their side-effect profile. It is presumed that the IP receptor-selective agonists will have fewer adverse events than those agents which activate multiple receptors although this remains to be determined clinically. For the remainder of this chapter, the physiological and pathophysiological context of these distinct membrane receptor profiles for the different prostacyclin mimetics will be evaluated. Comparative prostanoid receptor expression profiles in normal and diseased lung will be described and how this has the propensity to affect the mode of action of prostacyclin mimetics in arteries and veins and other cell types in PAH. We will also focus on the importance of EP_2 and EP_4 receptors as negative modulators of pulmonary artery smooth muscle tone, cell proliferation and fibrosis, and how these receptors alongside DP_1 receptors may have a specific role in the veins and the airways. Other data suggests that limiting EP_3 receptor activation will be beneficial in PAH as this receptor is upregulated by hypoxia and activates TGF-β signalling and may thus oppose signalling through BMPR2 receptors.

5.3 Prostanoid Synthesis and Receptor Expression

Prostanoids are derived from cyclooxygenase isoenzymes (COX-1 and COX-2), which convert arachidonic acid to an unstable intermediate, prostaglandin H_2, the common precursor for five principal bioactive derivatives. TXA_2, prostaglandin $F_{2\alpha}$,

prostaglandin E_2 (PGE_2), prostacyclin and prostaglandin D_2 (PGD_2) are then generated by the action of individual prostaglandin synthase enzymes. Once generated, the different prostanoids exert their pharmacological and biological effects by binding to and activating specific membrane bound G-protein-coupled receptors (Fig. 5.1). EP_1, EP_3, FP and TP receptors are contractile receptors coupled to Gq and Gi that either elevate intracellular $Ca^{2+}$and/or reduce cAMP, respectively. EP_2, EP_4, IP and DP_1 receptors are vasorelaxant receptors coupled to Gs that will activate adenylate cyclase and elevate intracellular cAMP (Fig. 5.1). The DP_2 (CRTH2) receptor, which has no significant homology to other prostanoid receptors, recognises PGD_2 as its endogenous ligand and is involved in allergic or T-helper 2 cell inflammation, with antagonists currently being trialled as a novel therapy for asthma. As the receptor is not activated to any significant extent by prostacyclin mimetics [48], for this reason it will not be further discussed.

As mentioned already, prostacyclin analogues have diverse effects on prostanoid receptors, with different IP receptor agonists likely to activate EP_1 (iloprost), EP_3 (prostacyclin) and EP_2 or DP_1 (treprostinil) receptors within their therapeutic dose range [6]. TP receptor activation by prostacyclin might come into play if either the plasma concentration is in excess of 50 nM (twice the reported plasma concentration in patients [49]), the receptor becomes primed by other vasoconstrictor agents (cross-sensitization), and/or its activity increases because the receptor becomes uncoupled from IP receptors, dimerization of which normally limits the deleterious effects of thromboxane [6]. Thus in order to understand the mode of action of these drugs in the therapeutic setting, it is important to assess the relative expression, function and impact of PAH on prostanoid receptors in small arteries and veins, the major sites of vessel disease. We have, therefore, sought to assess their expression in lung sections from control and PAH patients with end-stage disease using methods previously described [44]. For comparison, bronchial sections were examined and acted as a positive control since the staining for IP, $EP_{1–4}$ and DP_1 was strong to moderately strong for all of these receptors in the respiratory epithelium (Fig. 5.2).

5.3.1 Bronchial Smooth Muscle

IP, EP_2 and EP_3 receptor staining was strong in the bronchial smooth muscle, with EP_2 being the strongest (Fig. 5.2a). DP_1 staining was moderately strong, and EP_4 staining less uniform, but nonetheless the smooth muscle stained positively, while overall EP_1 receptor expression was weak. In studies conducted nearly 20 years ago, IP, EP_2 and to a lesser extent DP_1 receptors, but not the EP_4 receptor, were reported to be associated with human bronchus relaxation as assessed by prostacyclin analogues, PGE_2 and PGD_2 [50]. Furthermore, PGE_2 did not induce relaxation in tracheal smooth muscle preparations from EP_2 receptor knockout mice [51], consistent with these earlier observations of a dominant role for EP_2 receptors in regulating airway smooth muscle tone. However, more recent studies using highly specific receptor agonists and antagonists suggest that the EP_4 receptor is the main receptor subtype to promote relaxation to PGE_2 in human small airways [52]. However, in

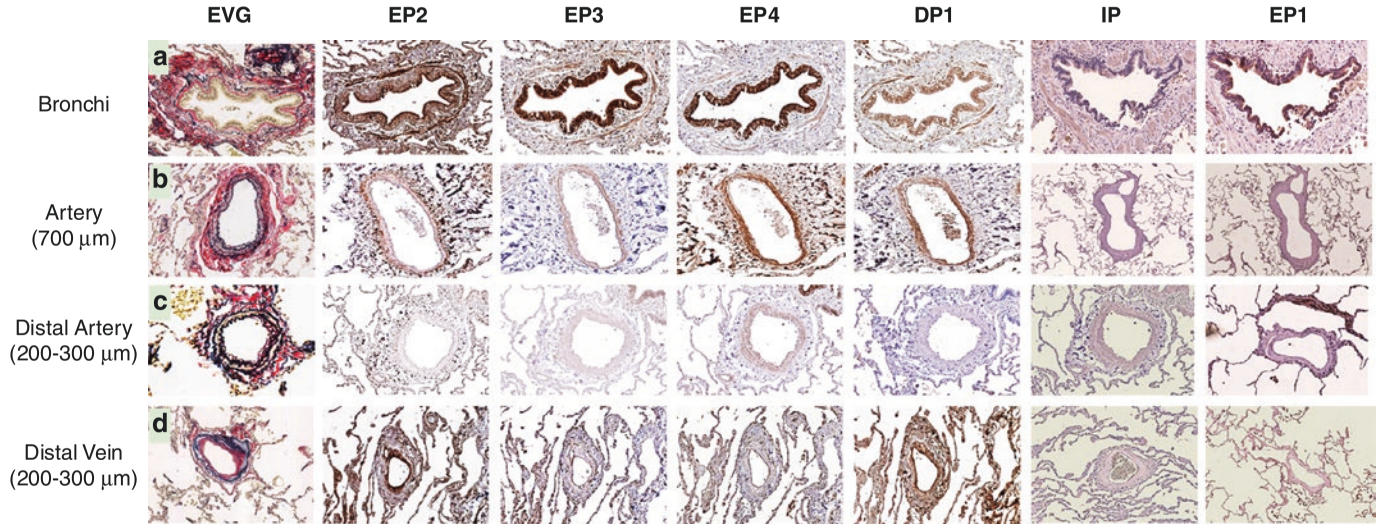

Fig. 5.2 Prostanoid EP_{1-4}, DP_1 and IP receptor expression in a human lung section from a control patient. Immunohistochemical staining was carried out in 10 µm sections of human lung tissue from a control patient. Each row from A to D represents sections of pulmonary bronchi, large artery (700 µm), distal artery (200–300 µm) and distal veins (200–300 µm), respectively. Far left panel, Van Gieson stain (EVG) shows collagen in red and elastic fibres in black. This was followed by antibody staining for different prostaglandin receptors: EP_2, EP_3, EP_4, DP_1 IP and EP_1 (from left to right columns) visualised by diaminobenzidine (brown) in sections counterstained with haematoxylin. All receptors are heavily expressed in the respiratory epithelium and act as a control for antibody staining in the arteries and veins

the same study, EP_2 receptors were reported to inhibit mast cell-mediated bronchoconstriction, highlighting a novel and potentially important function of EP_2 receptors. Inhibition of mast cell function might be considered beneficial in the context of PAH. Increasingly mast cells are thought to promote vascular remodelling in lung disease, including in PAH, releasing a number of mediators like histamine, PGD_2, leukotrienes, pro-inflammatory cytokines and growth factors like TGF-β [53].

With respect to contractile prostanoid receptors in the bronchus, TP receptors are the predominant functional contractile prostanoid receptor and appear to mediate contractions induced by high concentrations of PGE_2 [52, 54]. The latter would fit in with the relatively weak expression of EP_1 receptors that we observed in the human bronchus muscle (Fig. 5.2a). Based on this pharmacology, and that neither treprostinil nor iloprost has significant activity at TP receptors, it is suggested that these agents will be effective bronchial dilators through the IP receptor. EP_4 receptors may contribute to iloprost-induced relaxation, though given iloprost has a Ki of 212 nM at this receptor [45], the inhaled drug would have to reach concentrations in excess of 100 nM in order to do so. This is highly unlikely given the upper plasma concentrations of iloprost in patients is ~1 nM [6]. DP_1 receptors are, however, likely to contribute to treprostinil-induced bronchial relaxation. As EP_2 receptors were the most highly expressed prostanoid receptor in the bronchus smooth muscle, this might indicate a significant role for these receptors although further work is required to understand their function in small bronchi.

5.3.2 Pulmonary Blood Vessels

5.3.2.1 Endothelium

There was moderate to strong expression of all prostanoid receptors in the endothelium of large and small pulmonary arteries (Fig. 5.2b, c) although the strongest expression was observed in small veins (Fig. 5.2d), suggesting the endothelium may be particularly important for maintaining the patency of blood vessels in the venous circulation. Few studies have investigated the involvement of human prostanoid receptors expressed on the endothelium and none in small pulmonary vessels. Moreover, studies have often assessed prostanoid effects on vascular tone in the presence of inhibitors of nitric oxide (NO) synthase and COX to prevent the release of endogenous prostanoids and NO (e.g. [55, 56]), thus masking the potential effect/role of the endothelium. Nonetheless, NO synthase inhibition was reported to augment the contractile function of PGE_2 in rat pulmonary arteries [57]. Furthermore, a significant dependency on endothelial-derived NO for relaxation induced by beraprost and treprostinil in rat and human pulmonary arteries has been reported [58, 59], which is likely to be associated in part by activation of the IP receptor. Curiously, both MRE-269 and iloprost show no endothelial dependence to their vasorelaxant actions in pulmonary arteries [58, 59], demonstrating a differential mechanism of these two prostacyclin mimetics compared to beraprost and treprostinil. The clinical significance of these findings is unclear but may suggest differences in the clinical efficacy of combination therapies based around potentiation of NO signalling (e.g. phosphodiesterase type 5 inhibitors).

5.3.2.2 Pulmonary Artery

There was expression of all prostanoid receptors in the medial layer of large arteries, though EP_1 receptor expression was moderate to weak, as was EP_2 receptor staining which was mainly confined to the outer medial layer (Fig. 5.2b). Our immunostaining data broadly support many of the previously published findings investigating receptor expression and contractile function in large (control) pulmonary blood vessels from humans. In arterial preparations, the IP receptor was found to be the predominant receptor involved in relaxation [55], while EP_3 and TP receptors were the main contractile receptors to counteract this [60]. In other studies, EP_4 and DP_1 receptors were found to be expressed in human pulmonary arteries whereas EP_2 and EP_1 receptors were not [19, 56]. This would generally fit with the moderate to strong IP, EP_3, EP_4 and DP_1 receptor staining, the generally weaker staining of the EP_1 receptor we observed in larger arteries coupled with the overall weak staining of EP_2 receptors that was recently reported in large and small pulmonary arteries [44].

5.3.2.3 Differential Prostanoid Expression in Distal Pulmonary Artery and Veins

There were some distinctive features of prostanoid receptor expression in small arteries (Fig. 5.2c) and veins (Fig. 5.2d) in control sections of lung tissue that are worth noting. Staining for EP_2 receptors was weak in the medial layer of arteries, but strong in veins, with a broadly similar picture for DP_1 receptors. By contrast, EP_3 staining was observed throughout the media in arteries but was generally confined to the outer layer of the vein juxtaposed with the adventitia although the endothelium was, however, strongly stained. Likewise, the EP_4 receptor antibody stained the medial layer of the distal artery, and greater staining was observed in the vein compared to EP_3. With respect to the IP receptor, staining appeared stronger in the artery compared to the vein, while the EP_1 receptor antibody moderately stained both small artery and vein.

5.3.2.4 Distal Pulmonary Veins

Previous experiments in human proximal veins have provided clear evidence that prostanoid IP, DP_1 and EP_4 receptors mediate relaxation to prostanoids whereas TP and EP_1 receptors mediate constriction [55, 56, 61]. The particularly strong expression of EP_2 and DP_1 receptors in the endothelium and medial layer of small veins suggest a functional role of these two prostanoid receptors in regulating venous tone. Such an observation may explain why PGE_2 and PGD_2 are strong relaxants of human proximal pulmonary veins constricted with phenylephrine whereas they fail to cause relaxation in pulmonary arteries under similar experimental conditions [55, 56]. DP_1 receptors are expressed at the protein level in veins and reported to underlie in part the venorelaxation to treprostinil [19]. This is consistent with the observation of only a partial dependence of the IP receptor in mediating relaxation to treprostinil in both human [19] and rat pulmonary veins [62]. Furthermore, 40% of relaxation to treprostinil in human vessels still remained in the presence of both a DP_1 and IP receptor antagonist, suggesting an additional mechanism contributing to relaxation [19], possibly via EP_2 receptors. Indeed, functional EP_2 receptors have been

reported in large human pulmonary veins, with the EP_2 agonist, ONO-AE1-259, causing relaxation, albeit at relatively high concentrations [56]. The prediction is that EP_2 receptors will play a greater role in small pulmonary veins, given its strong expression both in the endothelium and in the medial layers. To our knowledge, there have been no reports investigating the role of EP_2 receptors in small pulmonary vessels, so the functional consequence of these receptors requires investigation. Elsewhere in the vasculature, the main vasodilator response to PGE_2 appears to be driven by the EP_2 receptor as PGE_2 produces hypotension in wild-type mice compared to substantial hypertension in EP_2 null mice [63]. This suggests a major role for EP_2 receptors in regulating vascular tone, at least in mice.

In terms of the function of other prostanoid receptors, EP_4 receptor agonists (e.g. ONO-AE1–329) strongly relax human pulmonary veins, and these were partially inhibited by an EP_4 antagonist as were PGE_2-induced relaxations [56]. Surprisingly, iloprost relaxations in pulmonary veins (and also arteries) were insensitive to EP_4 receptor antagonists [19]. Thus the EP_4 receptor as a proposed target for iloprost-mediated vasodilation during therapeutic use in human PAH [64] is so far not supported experimentally. Whether this relates to iloprost acting at various receptor targets (IP, EP_4 and PPARs) to cause relaxation, thus requiring the simultaneous treatment with multiple antagonists to inhibit functional responses is unclear, but supported by experimental observation in other vascular tissues [65]. EP_4 activation may be thwarted by the potent actions of iloprost at EP_1 receptors. Indeed EP_1 antagonists enhance iloprost and PGE_2 relaxation in human pulmonary veins and also underlie contractions seen by these two agents in the same tissue [55, 56, 61]. Likewise, the presence of an EP_1 antagonist was required before the relaxant effect of prostacyclin in pulmonary veins approached that in pulmonary arteries [66]. Furthermore, beraprost strongly relaxed human pulmonary arteries but not veins [19] whereas treprostinil was a stronger venorelaxant than an arterial relaxant in rat distal pulmonary blood vessels [62], presumably through its combined action at IP, DP_1 and EP_2 receptors. Such findings might provide a rationale for investigating the effects of treprostinil in pulmonary hypertension (PH) associated with post-capillary disease, of which PH due to left heart dysfunction is characterised predominantly by venoconstriction and remodelling (see later).

5.3.3 Prostanoid Receptor Expression in PAH

In lung sections derived from a patient with PAH, prostanoid receptor expression in the bronchi remained largely unchanged, the exception being the DP_1 receptor, expression of which went up in the bronchial smooth muscle (Fig. 5.3a). In contrast, there were marked changes to expression in pulmonary blood vessels (Fig. 5.3b–d). There was a reduction in the expression of all prostanoid receptor subtypes in the endothelium of arteries and veins, with IP and EP_1 receptor staining barely evident in small arteries, while EP_3 receptor expression appeared particularly reduced in the vein. Some loss of staining is undoubtedly due to endothelial damage which was apparent in many blood vessels. The pattern of prostanoid receptor expression

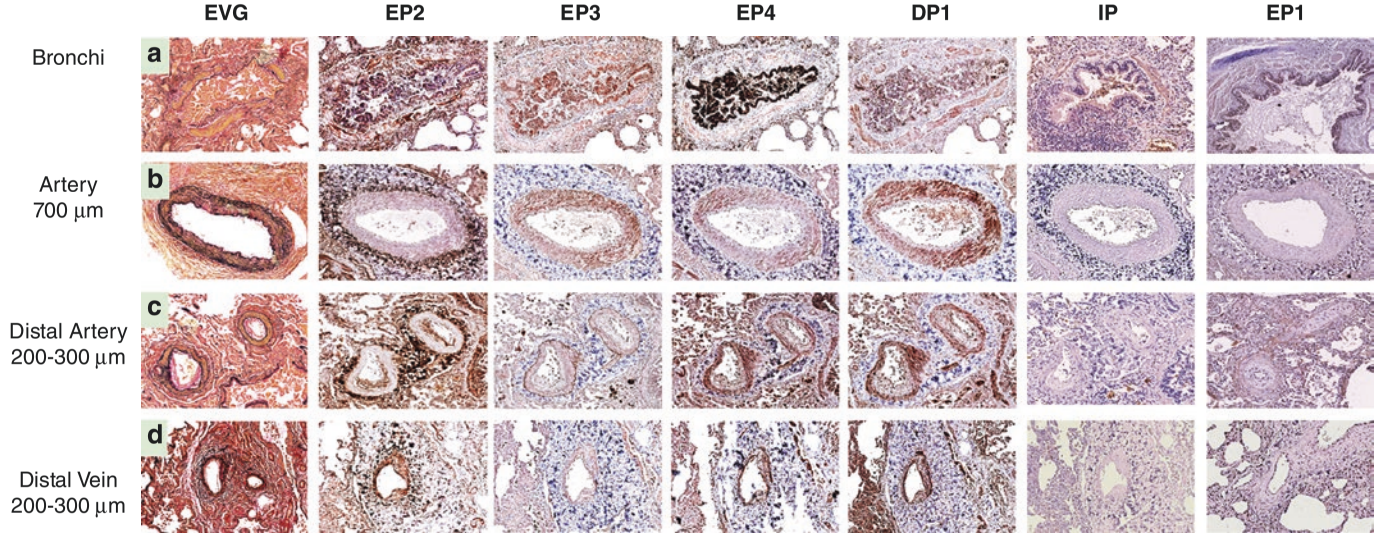

Fig. 5.3 EP_{1-4}, DP_1 and IP receptor expression in a human lung section from a PAH patient. Immunohistochemical staining was carried out in 10 µm serial sections of human lung tissue from patient with pulmonary arterial hypertension (PAH). Each row from **a** to **d** represents a sections of pulmonary bronchi, large artery (700 µm), distal artery (200–300 µm) and distal vein (200–300 µm) respectively. Far left panel, Van Gieson stain (EVG) shows collagen in red and elastic fibres in black. This was followed by antibody staining for different prostanoid receptors: EP_2, EP_3, EP_4, DP_1 IP and EP_1 (from left to right columns) visualised by diaminobenzidine (brown) in sections counterstained with haematoxylin

was somewhat different in smooth muscle. Prostanoid EP_3 receptor staining was increased in the medial layer of large and small arteries and in the vein compared to control, while EP_4 and EP_2 receptor antibody staining was increased in small arteries and veins. Similar to EP_2 and EP_4 receptors, DP_1 receptors were found to be strongly expressed in the small artery and vein. In contrast, IP receptor expression was weak in arteries and veins. Finally, the expression of EP_2 receptors in the adventitial layer of both large and small arteries and veins was marked, being particularly strong in PAH lung sections as was the case for the EP_4 receptor in small arteries (Fig. 5.3c). Staining in the adventitial layer is likely to come from fibroblasts, which preside predominately in this layer, undergoing proliferation in PAH and producing significant amounts of collagen to increase adventitial thickness [67, 68]. This was evidenced by the Van Gieson (EVG) stain which showed extensive collagen deposition (red staining). We cannot rule out the possibility that EP_2 and EP_4 receptor staining comes from inflammatory cell types, particularly monocytes and dendritic cells, which can be found in the adventitia of remodelled arteries in PAH [4, 67] and which express functional EP_2 and EP_4 receptors [60, 69]. Whether EP_4 receptors can account for any of the IP receptor-independent effects of iloprost on cell growth in PASMCs from PAH patients, where EP_4 function may be upregulated, requires investigation.

5.3.3.1 Downregulation of IP Receptors in PAH

It was not unexpected to find that IP receptor expression was downregulated in the arteries and veins in PAH, as previous studies have shown that mRNA and protein levels are substantially reduced both in the lungs and in PASMCs derived from PAH patients compared to controls [22, 64]. Furthermore, in a rat monocrotaline model of PAH, IP receptor mRNA levels were decreased while EP_4 and EP_2 receptor expressions were preserved [64], although in another study using the same model, no decrease in the IP receptor expression was found [33]. In a sugen/hypoxia model of severe PAH, the IP receptor was minimally expressed in whole lungs, and iloprost did not reduce mean PAP despite improving cardiac output [70]. This might suggest that in severe disease IP receptor function in the pulmonary circulation may be limited or be curtailed by the activation of prostanoid vasoconstrictor pathways opposing signalling through this receptor. That IP receptors were still functional in cultured PASMCs from PAH patients and significantly contributed to the anti-proliferative effects of non-prostanoid IP agonists may, however, be counterintuitive. It might be expected that due to the substantive drop in IP receptor expression found even in cell culture, combined with cells having been exposed to long-term prostacyclin therapy (epoprostenol or iloprost) for several months prior to isolation [22, 44], this would lead to substantive receptor desensitisation. Because this did not happen, it might suggest either a large IP receptor reserve (redundancy) in the system and/or that IP function recovers sufficiently well in culture before agonist treatment. Other studies have suggested that partial agonism leads to limited β-arrestin recruitment and less IP receptor internalisation, and hence a lower likelihood of receptor desensitisation with MRE-269 compared to other analogues [38]. Against this notion, MRE269 still remains the weakest anti-proliferative agent when compared to other prostacyclin analogues in the same assay [37].

5.3.3.2 Robust Expression of EP_2 and EP_4 Receptors in PAH: Key Anti-Fibrotic Targets

A striking difference in the pattern of prostanoid receptor mRNA expression was recently reported in human PASMCs derived from control versus PAH patients [44]. While prostanoid IP, EP_2 and EP_4 receptor mRNA levels were similarly expressed in control cells, the relative expression of EP_2 or EP_4 over the IP receptor was enhanced 84- and 15-fold, respectively, in PAH cells, suggesting that signalling through these receptors may be well-maintained or even upregulated in PAH [44]. Indeed, EP_2 and EP_4 receptors, as evidenced by the use of butaprost (selective EP_2 agonist) and iloprost, respectively, were functionally coupled to cAMP generation in PASMCs cells derived from either patients or rats with PAH [44, 64]. Moreover, using the selective EP_2 receptor antagonist, PF-04418948 [71] as well as EP_2 receptor siRNAs, the anti-proliferative effects of treprostinil were found to be mediated by this receptor over a wide concentration range (0.1–1000 nM) in human PASMCs from PAH patients [44]. This corroborates with an earlier observation that an IP receptor- and adenylate cyclase-independent mechanism was largely responsible for the anti-proliferative effects of treprostinil in the same diseased cell type, and appeared also to be the case for iloprost [22]. With respect to treprostinil, PPARγ plays a significant role in mediating the anti-proliferative effects of treprostinil in human PASMCs isolated from PAH patients [22] and by inference involves the EP_2 receptor in its activation although this remains to be established. Other PPARs may contribute to the anti-proliferative effects of beraprost [72] though this has not been investigated in PASMCs.

From a pharmacological perspective, this switch towards signalling via the EP_2 receptor with treprostinil may with hindsight not be surprising. Based on the high relative EP_2/IP receptor expression (84-fold) and that treprostinil has a tenfold greater affinity at the EP_2 receptor compared to the IP receptor [45], it suggests that it is >800× more likely to activate the EP_2 over the IP receptor in PAH cells. This does not necessarily mean that the IP receptor would not be activated by treprostinil at therapeutic doses as IP receptor activation could be observed under conditions of substantial EP_2 antagonism [44]. The situation is in stark contrast to the mode of action of the non-prostanoid IP agonists, MRE-269 and ralinepag, where the IP receptor appears to be entirely responsible for their anti-proliferative properties in cells from PAH patients assessed under similar experimental conditions [37, 44]. At this stage it is unknown whether non-prostanoid IP agonists can bind to PPARs and act in concert with the IP receptor [73] to promote their biological effects, but this is key information to ascertain.

The observation that EP_2 receptor expression was abundant relative to other prostanoid receptors may imply that it is upregulated as a consequence of disease. In this respect, EP_2 receptor expression can be enhanced in response to platelet-derived growth factor [74, 75] and transforming growth factor β [76], key drivers of smooth muscle proliferation in PAH [4]. Moreover, significantly more EP_2 receptors per cell (but not EP_4) were detected in asthmatic airway smooth muscle cells compared with non-asthmatic cells, and were found to be associated with enhanced sensitivity to the anti-proliferative effects of both PGE_2 and butaprost but not to either

prostacyclin or forskolin [74]. Likewise, EP_2 (and EP_4) receptor expression was enhanced in lung fibroblasts derived from patients with chronic obstructive pulmonary disease [77, 78]. Such observations are consistent with enhanced EP_2 receptor function/activity in airway remodelling as well as in PAH. While the role of the EP_2 receptor in the context of remodelling in PAH is unknown, it should be noted that the activation of EP_2 receptors has a range of inhibitory effects on fibroblast function that could have beneficial effects in PAH. Treatment of human lung fibroblasts with either PGE_2 or butaprost inhibits migration [79]. In addition, PGE_2 inhibits the transition of fibroblasts to myofibroblasts [80] and is capable of suppressing fibroblast proliferation and collagen synthesis through EP_2 receptor-mediated pathways [81]. Indeed, anti-fibrotic properties of treprostinil have been reported both *in vivo* (bleomycin-induced pulmonary fibrotic model) and *in vitro* (inhibition of TGF-β-stimulated collagen production), though the receptor involved was not defined in these studies [82, 83]. It should also be mentioned that some of the above effects can be replicated by EP_4 receptors [47, 84], suggesting some overlapping function of EP_2 and EP_4 receptors in regulating fibroblast function. Iloprost appears to ameliorate established cardiac fibrosis by preventing collagen synthesis and increasing collagen turnover, and though the receptor mechanism was not specifically established in this study, evidence was presented that the IP receptor was probably not involved [70]. It is worth noting that in a high-throughput screen to assess the therapeutic potential of novel drugs in pulmonary fibrosis, drugs acting at either the EP_2 or EP_4 receptor were identified as amongst the most effective agents at inhibiting TGF-β-induced myofibroblast differentiation via the SMAD2/3 pathway [85].

5.3.3.3 EP_3 Receptors May Contribute to Disease Pathology in PAH

It is important to understand the role of contractile prostanoid receptors as these could limit the doses of prostacyclin mimetics given therapeutically or potentially give rise to unwanted side effects. Of note, EP_3 expression went up in small arteries from PAH patients, as has been reported in the airway smooth muscle cells derived from asthmatic patients compared to controls [74]. Likewise, enhanced EP_3 expression was observed in rodent models of PAH [33, 86] and was associated with increased sensitivity to the EP_3 agonist, sulprostone, suggesting a gain of function at this receptor [33]. Elevated EP_3 receptor expression would be predicted to dampen cAMP signalling in cells and hence potentially reduce prostanoid-induced relaxation in pulmonary blood vessels as well as relaxation to non-prostanoid IP agonists. Prostacyclin action may be curtailed since it potently binds to the EP_3 receptor (Ki of ~10–40 nM) and the plasma concentration in patients (~25 nM) would be in the range for receptor activation [6, 49]. Many studies have provided evidence that the contractile effects of prostacyclin analogues are mediated through EP_3 receptors [33, 58, 65, 87] and that their inhibition can enhance vasorelaxation induced by these agents [33, 65]. However, based on binding affinities, EP_3 receptors are unlikely to be activated by prostacyclin analogues in the clinical setting; the upper plasma concentration achieved in humans is ~1 nM for iloprost and ~50 nM for treprostinil which is 50× the Ki for both drugs [6]. Consistent with this interpretation, the contractile effects of prostacyclin analogues are only observed at high concentrations (≥1000 nM) in distal rat pulmonary arteries

although priming of the tissue with phenylephrine was required before contractions to treprostinil were even observed [87]. Thus any counteracting effect of EP_3 is likely to arise from basal activity of the receptor and not from direct activation by prostacyclin analogues. EP_3-TP-induced vasoconstrictive synergism has been described in blood vessels where priming with a TXA_2 mimetic markedly increases both the potency and size of contraction to EP_3 agonists (see [87]). Enhanced synergy between EP_3 and TP receptors could occur in PAH because of increased TXA_2 levels [88] and because of enhanced activity of EP_3 receptors. Such an interaction might provide an explanation of why iloprost showed far less relaxation and sensitivity in human pulmonary arteries when contracted with the TXA_2 mimetic (U46619) as opposed to phenylephrine [87]. This might suggest that the vasodilatory responses of prostacyclin and iloprost in the lung may be more adversely affected in patients with high TXA_2 levels compared to other IP receptors agonists which either have less sensitivity towards the EP_3 receptors or do not activate them.

EP_3 receptors may contribute to disease pathology in PAH as inhibition of EP_3 receptors either pharmacologically or through deletion of smooth muscle EP_3 receptors attenuated right ventricular systolic pressure (RVSP) and vascular wall thickness in rats with mild (chronic hypoxia), moderate (monocrotaline) or severe (sugen/hypoxia) PAH [86]. Interestingly, the mechanism appeared related to hypoxia-induced TGF-β activation via Rho-associated kinase, with the downstream activation of SMAD2/3 leading to extracellular matrix remodelling via upregulation of collagen 1, fibronectin and tenascin-C genes. Effects were, however, independent of cell proliferation [86], so this suggests that the main role for the EP_3 receptor is to module vascular tone and stiffness in PAH through TGF-β-induced changes in extracellular matrix remodelling.

5.3.3.4 Role of the Veins in PAH and Other Classified Groups of PH

The extent to which venous remodelling contributes to the pathology in PAH is currently a subject of much debate [89] and is thwarted by the lack of good venous biomarkers and the difficulty in identifying veins that might masquerade as a remodelled artery. We can functionally distinguish distal arteries from veins on the basis of their differential response to PGD_2 and PGE_2 in blood vessels pre-contracted with U46619 (thromboxane mimetic). The former relaxes veins while having little effect on tone in arteries, contrasting with PGE_2, which has potent contractile effect in arteries but is minimally contractile in veins under similar experimental conditions (unpublished observations). Whether this will be observed in remodelled veins and arteries is unknown but should be investigated. Strong expression of DP_1 and EP_2 and EP_4 receptors in veins suggests this pattern of response may be preserved.

Elucidating the role of venous remodelling in PH and what might be an effective treatment strategy is complicated by their being a spectrum of disease. This might be seen as a continuum from pure pre-capillary disease (perhaps defined as IPAH) to secondary causes giving rise to a mixed pre- and post-capillary disease (e.g. CTD) through to a predominantly post-capillary phenotype, epitomised by PH due pulmonary venoocclusive disease or left heart disease. Interestingly, increased pulmonary venous remodelling has recently been demonstrated in PAH patients harbouring

BMPR2 mutations compared to those without, showing a somewhat similar histological presentation to inoperable chronic thromboembolic pulmonary hypertension (CTEPH) with persistent PH, i.e. fibrotic lesions associated with bronchial to pulmonary shunting [90, 91]. In the context of PH, targeting the veins is important as remodelled veins will significantly contribute to PVR, and mismatching (dilation in arterial but not venous side) will cause higher sheer stress in veins and increase PAP. Enhanced collagen deposition, which makes veins less distensible, would also contribute to the elevated pressure. In primary PH (IPAH), intimal and adventitial thickening of pulmonary veins <250 μm was estimated to be observed in ~50% of cases [92]. Given this study predates the discovery of BMPR2 mutations in the IPAH population [8, 11], and that the current definition of IPAH has a more stringent hemodynamic definition (lower mean PAP and pulmonary artery wedge pressure), suggests that this figure might be overestimated and should thus be re-evaluated [93].

In humans, pulmonary veins have a greater vasoconstrictor sensitivity towards ET-1, TXA_2, leukotrienes and platelet activating factor (PAF) than in arteries, a finding reported in many animal species as well [94]. In addition, numerous studies have shown intense constriction of pulmonary veins in response to hypoxia in a variety of species which may involve the release of ET-1 and TXA_2 [94]. The consequence of venous constriction will be to increase pulmonary capillary pressure and thus promote pulmonary oedema. ET-1 is known to increase capillary permeability via post-capillary vasoconstriction, which is also dependent on the presence of inflammatory cells [95]. Experiments in EP_3 receptor null mice suggest that the EP_3 receptor is responsible in part for oedema triggered by PAF in rat lungs [96] and, as discussed above, is also linked to enhanced collagen production [86]. It also suggests that hypoxia in the veins may be an underlying trigger for oedema via this prostanoid receptor.

The potential for the deleterious effects of prostacyclin (epoprostenol) in patients with severe congestive heart failure and POVD, and the failure of agents that increase cGMP (riociguat and sildenafil) to show significant improvement in the context of PH with left heart disease, has led to the belief that vasodilatory therapy is not appropriate or at least controversial in this setting [89, 97, 98]. Several factors may account for the problems faced with epoprostenol use in patients with diagnosed venous disease. Not only is this agent predicted to be a poor venodilator in humans [19], due in part to its actions on the EP_1 receptor in veins [66], but also the activation of the EP_3 receptor is likely to cause oedema and perpetuate vasoconstriction [94]. The latter may help to explain why pulmonary oedema is commonly reported in POVD patients on epoprostenol therapy [98], particularly in those patients on higher doses of the drug, which would give rise to significant activation of the EP_3 receptor. In severe venous disease, the hypoxic environment coupled with high TXA_2 levels and low IP receptor function might actually be predicted to convert prostacyclin into a venoconstrictor, which would have detrimental consequences (c.f. [97]). For similar reasons, the use of iloprost and beraprost may also be contraindicated in PH-related venous disease. Both, particularly beraprost, are better arterial dilators than venodilators in human pulmonary blood vessels. Iloprost has potent actions on the contractile EP_1 receptor [45] which is functionally

expressed in veins, while repeated administration with beraprost in mice has been reported to increase circulatory TXA_2 levels [24]. Whether such an effect occurs in humans remains to be determined, but this might explain why beraprost is a particularly poor venodilator and why effects may not be sustained in PAH patients [1]. In contrast, MRE-269, which is devoid of significant EP_3 receptor activity, is surprisingly a slightly better veno than an arterial vasorelaxant, the significance of which remains unclear but might suggest some usefulness in treating venous disease.

The pharmacological profile of treprostinil suggests it should be seriously considered as a treatment option in PH associated with venous remodelling. Its ability to activate IP, DP_1 EP_2 receptors coupled to the robust expression of the latter two receptors in PAH suggests that its use could provide a beneficial response both as a venodilator and as an anti-fibrotic agent even in the absence of the IP receptor. As such, the efficacy of oral treprostinil, in a multi-centre randomised double blinded trial, is currently being investigated in patients with PH in heart failure with preserved ejection fraction (TDE-HF-301). On a cautionary note, in a retrospective analysis of a PAH cohort (1996–2006) with high rate of contamination from post-capillary disease (of different aetiologies), patients with a mean pulmonary artery wedge pressure (PAWP) >12 did not show meaningful changes in PVR or PAP whereas those classified as having 'pure' pre-capillary disease did [93]. This occurred despite patients having a significant increase in cardiac output and a placebo-corrected improvement in 6-min walk test of 35 m compared to 23 m in the IPAH group. Interestingly, in another randomised study of non-operable CTEPH or persistent PH pulmonary hypertension, patients did respond to treprostinil with significant hemodynamic improvement, including in PVR and mean PAP [99]. The extent to which these patients had venous disease was not reported, but it is possible that venous improvement may have occurred.

5.4 BMPR2 and TGF-β Signalling in PAH and Impact of Prostacyclin Analogues

Since the original discovery of a loss-of-function mutation in the gene encoding for BMPR2 being associated with PAH, more than 300 disease-causing mutations have been described throughout the BMPR2 gene, including critical regions such as the kinase domain [8, 11]. BMPR2 is a transmembrane serine-threonine kinase, which serves as a receptor for bone morphogenetic proteins (BMPs). In association with a co-receptor, usually BMPR1A, BMPR2 can signal through multiple pathways, including the canonical Smad1/5/8-dependent pathway (Fig. 5.4) [5, 6, 8]. The impact of prostacyclin analogues on BMPR2 signalling was investigated by Yang and colleagues in 2010 [9]. They found that iloprost and treprostinil enhanced BMP-induced phosphorylation of Smad1/5 and caused induction of the inhibitor of DNA-binding protein 1 (Id1), both of which are downstream effectors of BMPR2 in normal PASMCs (Fig. 5.4). The growth-suppressive effects of BMPs were re-established in PASMCs derived from patients harbouring BMPR2 mutations without restoring BMPR2 protein levels, consistent with a mechanism that

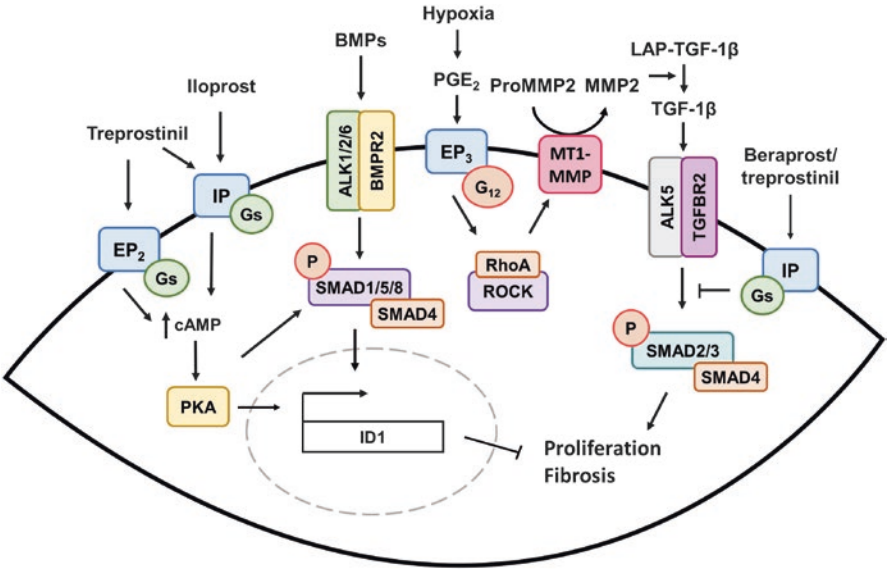

Fig. 5.4 Prostacyclin analogues can enhance bone morphogenic protein receptor type 2 (BMPR2) signalling and oppose cell growth induced via the transforming growth factor beta 1 (TGF-1β) receptor pathway. Upon ligand binding, the BMPR2 receptor phosphorylates a type I receptor (ALK1, ALK2, or ALK6) leading to the phosphorylation of SMAD 1/5/8. The subsequent phosphorylation of SMAD 4 causes translocation of the phosphorylated SMADs to the nucleus to modulate the expression of target genes that inhibit pulmonary artery smooth muscle cell (PASMC) growth and promote apoptosis. Iloprost and treprostinil enhance BMPR2-mediated SMAD 1/5/8 phosphorylation and inhibitory DNA binding protein 1 (ID1) transcription by recruiting IP and EP_2 receptors and activating cAMP-dependent protein kinase A (PKA); induction of ID1 expression can also occur independently of SMADs. Activation of EP_3 receptors by hypoxia in PASMCs pulmonary leads to Rho-dependent activation of metalloproteinase type 2 (MMP2), with the release of active TGF-1 β and the downstream activation of SMAD2/3 via TGF-1β type II receptor (TGFBR2), which phosphorylates a type I receptor, ALK5. The consequence will lead to enhanced proliferation and collagen deposition

bypasses the defective receptor. The prostanoid receptor involved remains undetermined although prostacyclin analogue effects on BMPR2 signalling were shown to be cAMP/PKA-dependent. Likewise, the phosphodiesterase type 5 inhibitor, sildenafil, another PAH therapy, was able to enhance BMPR2 signalling either in normal or mutant cell PASMCs by acting through cyclic GMP-dependent protein kinase I [100]. Thus, prostacyclin drugs known to act by increasing NO (beraprost, epoprostenol and treprostinil) might be predicted to promote a more robust activation of this pathway, with or without sildenafil.

In addition to a deficit in Smad1/5 phosphorylation, heightened Smad2/3 phosphorylation and transcriptional activity have been demonstrated in BMPR2-mutant PASMCs, indicative of increased pro-proliferative TGF-β signalling unopposed by growth-suppressive BMPR2 signalling [101]. Moreover, in contrast to normal PASMCs, upon which TGF-β has growth-suppressive effects, TGF-β promotes the proliferation of BMPR2-mutant PASMCs. The prostacyclin analogue beraprost

inhibited TGF-β-induced Smad2/3-dependent signalling (Fig. 5.4) and was more effective at inhibiting the proliferation of BMPR2 mutant PASMCs than that of their wild-type equivalent. Beraprost, like iloprost and treprostinil, could, therefore, be rescuing the diminished BMRP2 signalling while inhibiting the augmented TGF-β signalling to reduce the proliferative capacity of BMPR2-mutant PASMCs. Such a mechanism may also be important in the heart where beraprost was shown to inhibit rat cardiac fibroblast proliferation by activating IP receptors and suppressing the TGF-β/Smad signalling pathway [102].

5.5 Regulation of TASK-1 By Prostacyclin Mimetics: Implications in PAH

Two-pore domain potassium channels of the KCNK gene subfamily, designated K_{2P} channels, mediate leak or background potassium currents (I_{KN}) and underlie the high-membrane permeability to K^+ at physiological resting membrane potentials [103]. KCNK3 encodes a TWIK-related acid-sensitive potassium channel, also known as TASK-1, and is the only TASK subunit to be expressed in either normal or PAH patient lungs [104]. In many cell types, including PASMCs, extracellular acidosis strongly and reversibly inhibits TASK-1 currents and causes membrane depolarisation whereas extracellular alkalosis enhances the currents mediated by TASK-1 to hyperpolarise the membrane. Several lines of evidence suggest a key role for TASK-1 in regulating pulmonary vessel diameter and, hence, vascular tone through its sensitivity to vasoactive mediators, pH, membrane stretch and hypoxia [105, 106]. TASK-1 currents are switched off by hypoxia in PASMCs and are proposed to contribute to standard terminology in the field [106].

In 2013, using whole-exome sequencing, novel heterozygous mutations in the KCNK3 gene were identified in patients with IPAH and HPAH [107]. Subsequently, homozygous KCNK3 mutations were later reported in patients with very aggressive and early-onset forms of PAH [108]. All KCNK3 mutations have severely reduced whole-cell TASK-1 currents recorded from COS-7 as well as from PASMCs expressing both wild-type and mutant TASK-1 subunits to reproduce the heterozygosity observed in carriers of the mutations [104, 107]. Moreover, TASK-1 expression levels were found to be markedly reduced in the explanted lungs of patients with IPAH but lacking disease-causing KCNK3 mutations [109], suggesting more widespread dysfunction of TASK-1 in PAH. Consistent with this notion, rats chronically treated with A293, a selective blocker of TASK-1 channels, were shown to develop early manifestations of PAH, including muscularisation of distal pulmonary arteries (normally non-muscular) and elevated RVSP [109]. Histological analysis of pulmonary arteries from these A293-treated rats revealed marked proliferation of pulmonary arterial endothelial cells (PAECs), PASMCs and pulmonary adventitial fibroblasts [109]. Moreover, TASK-1 currents were also shown to be attenuated in PAECs and PASMCs derived from the lungs of patients with HPAH due to BMPR2 mutations, suggesting that TASK-1 channel

downregulation might represent a second hit required for PAH to manifest in carriers with BMPR2 mutations [109].

Activation of different potassium channel types represents an important mechanism underlying the vasorelaxing properties of prostacyclin analogues, including large-conductance calcium-activated, ATP-sensitive and TASK-1 potassium channels [6, 110]. TASK-1 appears to be a downstream target of the IP receptor where iloprost and treprostinil have both been shown to equipotently (EC_{50} 1 nM) enhance I_{KN} currents through TASK-1 channels in normal human PASMCs [110]. The effects of iloprost and treprostinil on TASK-1 currents were mimicked by 8-br-cAMP, a membrane-permeant form of the second messenger cAMP, and blocked by the protein kinase A (PKA) inhibitor, KT5720 and the IP receptor antagonist, RO1138452, suggesting that in healthy human PASMCs prostacyclin analogues signal primarily via the IP/cAMP/PKA pathway to increase TASK-1 currents (Fig. 5.5) [106, 110]. Consensus PKA phosphorylation motifs were also identified within TASK-1 and treprostinil was shown to promote the phosphorylation of these sites [106]. Interestingly, cAMP-dependent PKA was reported to promote

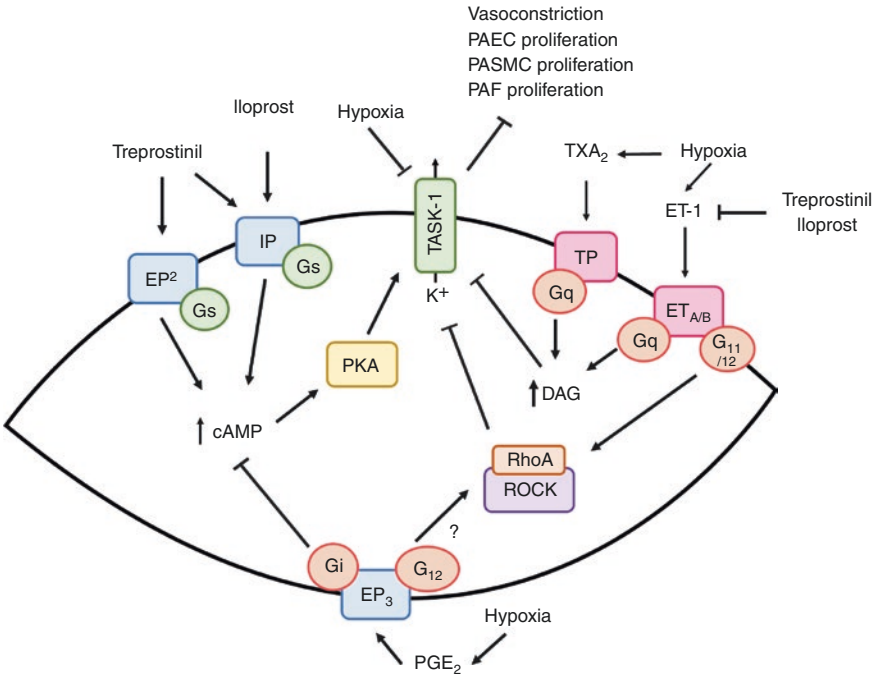

Fig. 5.5 Opposing regulation of TASK-1 by prostacyclin analogues and by hypoxia. TASK-1 in pulmonary arterial smooth muscle cells (PASMCs) is activated by prostacyclin analogues via the IP or EP_2 receptor, while EP_3 receptors oppose this action either through lowering cyclic AMP (cAMP) or via activation of the Rho pathway. Hypoxia-induced synthesis of thromboxane A_2 (TXA_2) or endothelin-1 (ET-1) will inhibit TASK-1 though activation of Gq or $G_{11/12}$. *DAG* diacylglycerol, *PKA* protein kinase A, *ROCK* Rho-associated protein kinase

the forward trafficking of TASK-1 channel subunits from the ER (where they are synthesised) to the plasma membrane where they assemble to form functional, current-conducting channels [111, 112]. Thus, treprostinil or iloprost could be increasing TASK-1 currents in human PASMCs by promoting the forward trafficking of TASK-1 channel subunits from the ER to the sarcolemma. Whether iloprost and treprostinil can rescue the depressed TASK-1 currents in PASMCs from patients with PAH, and whether these channels contribute to the anti-proliferative effects of prostacyclin analogues, remain to be established. Downregulation of the IP receptor in PAH could mean that iloprost becomes less effective at enhancing TASK-1 currents than treprostinil, which is also able to recruit the EP_2 receptor to enhance TASK-1 currents [113].

Several mechanisms may account for the depressed function of TASK-1 in PAH. Gq-coupled receptors, including those activated by ET-1, 5-HT and TXA_2, suppress current through TASK-1 channels (Fig. 5.5) [103]. Given that all these mediators would be elevated in PAH [5, 88, 114], this may lead to a generalised inhibition of the channel. Prostacyclin analogues are potent inhibitors of ET-1 synthesis, both in pulmonary endothelial and smooth muscle cells [6], so these agents could negate the inhibitory effects of ET-1 on TASK-1. The mechanisms by which hypoxia inhibits TASK-1 currents are unclear but may involve increased ET-1 production, given that ET-1 expression and secretion have been shown to be induced by hypoxia in pulmonary endothelial, smooth muscle cells and fibroblasts [94, 114]. ET-1 has been shown to reduce TASK-1 currents in PASMCs by recruiting the RhoA/Rho kinase pathway [115, 116]. EP_3 receptors have been shown to be upregulated in PASMCs in response to hypoxia and are directly linked to activation of the RhoA/Rho kinase pathway [86], so may, like ET-1, inhibit TASK-1 currents through this pathway (Fig. 5.5). EP_3 receptors also couple to $G_{i/o}$ to inhibit cAMP production and so could counteract the effect of prostacyclin analogues on TASK-1 currents, particularly under hypoxic conditions.

5.6 Prostacyclin Effects on Vascular Remodelling In Vivo: Outstanding Issues

The key question is whether prostanoid drugs used to treat PAH patients have positive effects on vascular remodelling associated with the abnormal proliferation of different cell types within the blood vessel wall. In vitro studies reported from a number of laboratories using vascular cells isolated from the lungs of patients with end-stage disease suggest that they should do so *in vivo* [9, 22, 83, 101]. Our own studies directly comparing the anti-proliferative properties of several clinically approved therapies in PASMCs from PAH patients showed hugely variable responses with the lowest dose to significantly inhibit cell proliferation varying some 10,000-fold from 0.1 nM for treprostinil up to 1 μM for the ET-1 receptor antagonist bosentan [117]. By inference, therapies are, therefore, likely to have vastly different effects on the remodelling process *in vivo* and pose challenges when designing protocols. Nonetheless, translation into positive effects on vascular remodelling has been

consistently reported in a monocrotaline model of PAH where treprostinil, beraprost and iloprost all significantly improved pulmonary haemodynamics as well as reducing muscularisation and the cell proliferation index of small blood vessels [9, 118–120]. This may in part be due to the analogue effects on inflammatory cell types like macrophages, dendritic cells and mast cells [6], which play a pivotal role in regulating pulmonary vascular remodelling in the monocrotaline model [121] and also in humans [53, 67, 89]. Dysfunctional BMPR2 signalling can also be rescued by prostacyclin analogues through their downstream enhancement of phosph-Smad1/5 and Id1 gene expression in monocrotaline-induced PAH in rats [9], which may act in concert with analogue effects on inflammatory cell types, particularly dendritic cells [6].

With respect to the Sugen 5416/hypoxia model of severe PH, which is a mixed model of group 1 (PAH) and group 3 (due to lung disease and/or hypoxia) PH, all analogues improved cardiac function either by reducing RVSP and RV hypertrophy (beraprost and treprostinil), by increasing exercise capacity and reducing cardiac fibrosis (iloprost) or by improving the ultrastructure of the heart [70, 119, 122]. This would fit with data suggesting that prostacyclins have positive effects on RV function in the heart [123]. Beraprost, given at a high dose as an inhaled bolus in nanoparticles (150 μg/kg), was the only analogue to reduce the muscularisation of arteries, possibly suggesting under dosing may have occurred in the other two studies [70, 119, 122]. The interpretation and significance of the above results would be greatly enhanced if the impact of Sugen 5416/hypoxia on prostanoid receptor and PPAR expression was known, and if hypoxia is a confounding factor in limiting the ability of prostacyclin drugs to activate any of their downstream targets. This could occur, for example, through the excessive production of TXA_2 and ET-1 within the blood vessel wall by hypoxia. This would lead to activation of TP receptors which may cause substantial IP receptor desensitisation and/or enhance EP_3 receptor activation and may thus affect some analogues (iloprost and prostacyclin) more than others. Rats have the peculiar property in that prostacyclin analogues do not relax pulmonary arteries constricted with ET-1 at all whereas they strongly relax human arteries [47]. Whether this means that these agents are not, therefore, able to reverse the mitogenic effects of ET-1 in rats should be considered. Dysfunctional BMPR2 signalling with depressed phospho-Smad1/5 and Id expression is a key feature of the monocrotaline-induced PAH model [9, 10] whereas surprisingly it does not appear to be a feature of the sugen/hypoxia model of PAH as reported in a recent study [122]. This represents a potentially key difference between how PAH is generated in these two classic models. Likewise, many drugs, including potent anti-cancer or anti-angiogenic drugs, do not reverse remodelling in sugen/hypoxia either [124], possibly suggesting that enhanced drug resistance is occurring in this experimental setting, for example, from the inactivation of regulators of the tumour suppressor gene, p53. The use of simvastatin in PAH is not supported by clinical trial data in patients [125] but is one of the very few drugs shown to reduce the number of obliterated lung vessels in the Sugen 5416/hypoxia model. This demonstrates the unpredictability of translating results from animal models into the clinical area.

Another reason why it may be difficult to translate prostanoid receptor function in rodents directly into humans is that, depending on the species, IP and EP_2 receptors can have pro- or anti-inflammatory actions in rat/mouse [126] and human [127] macrophages, respectively. This may confound the interpretation of prostacyclin action in animal models of PAH. The significance of the curious absence of perivascular infiltration of inflammatory cell types in the sugen/hypoxia model [128] needs to be addressed. Moreover, the rat expresses an atypical IP receptor which has a relatively low affinity for the selexipag metabolite compared to the human receptor [32], making it difficult to assess the properties of MRE-269 with other prostacyclin analogues. The same may be true in mice, the other species regularly used to model PAH disease. In this respect, there is no published data on prostanoid receptor affinities in the rat and mouse for treprostinil or much knowledge about prostanoid expression in the lung of these species and whether/how this differs to the pattern of expression in the human lung.

5.7 Future Work and Clinical Implications

Studies suggest that TASK-1 is likely to be a key target for prostacyclins either through the IP or EP_2 receptor. It will be important to understand the role of this channel in experimental PAH models, dysfunction of which is known to promote vasoconstriction, cell proliferation and resistance to apoptosis in cells [12]. Whether prostacyclins can rescue such dysfunction, and thus become a treatment strategy for those patients harbouring TASK-1 mutations, remains to be determined. Along the same lines, upfront prostacyclin therapy should be considered for patients with dysfunctional TGF-β1 and BMPR2 signalling, and those with venoconstriction or remodelling the use of treprostinil considered. Interestingly, in a small scale study in children, only epoprostenol appeared to provide significant improvement out of all the standard PAH therapies in patients habouring BMPR2 and ALK-1 mutations [129]. Furthermore, a number of patients were found to respond to oxygen therapy. It is tempting to speculate here that switching off of the hypoxic-sensitive EP_3 receptor, which is linked to TGF-β receptor activation, may provide some clinical benefit as might activating the hypoxia-sensitive TASK-1 channel. This remains an interesting area for further research.

We have little knowledge about prostacyclin signalling in human distal pulmonary vessels, and to our knowledge no studies have specially investigated vascular reactivity and remodelling processes in small veins in PAH. Clearly future experiments should focus on bridging these gaps. The diverse pharmacology of prostacyclin, its analogues and the newly developed non-prostanoid selective IP agonists potentially give each one of them advantages and disadvantages in different clinical scenarios that should be exploited by patient stratification of PAH and PH subtypes as well as from a personalised medicine approach. These agents are not just vasodilators; they are potent anti-proliferative agents in smooth muscle, and increasing evidence suggests that they act to dampen down fibrosis through their actions on the pro-fibrotic TGF- β signalling pathway, and some of this may also translate into long-term improvements of heart function. The inhibitory action of prostacyclins on platelet function should

not be underestimated in PAH, and having a selective IP agonist like selexipag, which is seemingly devoid of *in vivo* action on platelets, may help to tease out their role. From a clinical standpoint, treprostinil through its potent action on EP_2 and DP_1 receptors may provide another treatment option in those patients seen not to be responding well to IP receptor-selective agonists, who are subsequently identified with having low IP receptor expression or limited functional capacity of the receptor. Its pharmacological profile suggests it should also be a good venodilator and strong anti-fibrotic agent through its action on the EP_2 receptor.

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