The mechanistic basis of prostacyclin and its stable analogues in pulmonary arterial hypertension: role of membrane versus nuclear receptors

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Abstract

Pulmonary arterial hypertension (PAH) is a progressive disease of distal pulmonary arteries in which patients suffer from elevated pulmonary arterial pressure, extensive vascular remodelling and right ventricular failure. To date prostacyclin therapy remains the most efficacious treatment for PAH and is the only approved monotherapy to have a positive impact on long-term survival. A key thing to note is that improvement exceeds that predicted from vasodilator testing strongly suggesting that additional mechanisms contribute to the therapeutic benefit of prostacyclins in PAH. Given these agents have potent antiproliferative, anti-inflammatory and endothelial regenerating properties suggests therapeutic benefit might result from a slowing, stabilization or even some reversal of vascular remodelling in vivo. This review discusses evidence that the pharmacology of each prostacyclin (IP) receptor agonist so far developed is distinct, with non-IP receptor targets clearly contributing to the therapeutic and side effect profile of PGI_2 (EP_3), iloprost (EP_1), treprostinil (EP_2, DP_1) along with a family of nuclear receptors known as peroxisome proliferator-activated receptors (PPARs), to which prostacyclin and some analogues directly bind. These targets are functionally expressed to varying degrees in arteries, veins, platelets, fibroblasts and inflammatory cells, and are likely to be involved in the biological actions of prostacylins. Recently, a highly selective IP agonist, selexipag has been developed for PAH. This agent should prove useful in distinguishing IP from other prostanoid receptors or PPAR binding effects in human tissue. It remains to be determined, whether selectively for the IP receptor gives rise to superior or inferior clinical benefit in PAH.

1. **Introduction**

Pulmonary arterial hypertension (PAH) is a debilitating and fatal disease, involving extensive remodelling and narrowing of the blood vessels within the pulmonary vasculature. This leads to increased pulmonary vascular resistance and right ventricular hypertrophy, with eventual heart failure and death [1]. Without appropriate treatment, adults with PAH have a median life expectancy of 2.8 years from diagnosis while children have less than 10 months [2,3]. The disease is probably initiated by endothelial damage, caused by a combination of sheer stress, hypoxia and genetic factors (including mutations in the transforming growth factor family of genes) leading to increased production of vasoconstrictors (endothelin & thromboxane) accompanying the loss of vasodilator and anti-platelet agents, prostacyclin (PGI_2) and nitric oxide (NO). This process is exacerbated by ion channel dysfunction, including loss of potassium channel activity/expression (voltage-gated and the two-pore domain K^+ channels) and upregulation of calcium entry through canonical transient receptor potential cation (TRPC) channels both leading to smooth muscle membrane depolarisation and Ca^{2+} influx [4]. The earliest known pathology in PAH is medial thickening due to hypertrophy and hyperplasia. However, as the disease progresses, proliferation of adventitial and intimal layers take over and uncontrolled endothelial proliferation is thought to underlie plexiform lesions. High cell proliferation rates within all layers (adventitia, media and endothelium) coupled with decreased programmed cell death (apoptosis) is a widely accepted explanation for the structural changes seen in PAH [1,5-7]. Indeed proliferative rates of human pulmonary arterial smooth muscle cells (PASMCs) isolated from patients with idiopathic PAH (IPAH) and grown in culture are close to double that of normal cells suggesting a switch from a less contractile to a more proliferative cellular phenotype [8,9]. Moreover proliferative rates are much higher in paediatric versus adult IPAH cells, consistent with the particularly aggressive nature of this disease in children [9]. In addition PASMCs isolated from PAH

patients lose responsiveness to bone morphogenetic protein (BMP) ligands and display excessive proliferation in response to transforming growth factor β1 (TGF-β1), when typically both these agents have antiproliferative and apoptotic effects in normal cells [10,11]. Thus the disease appears to alter the intrinsic properties either of resident smooth muscle cells, or those cells which have become incorporated into the hyperplastic medial layer. One such event which may drive this is sustained hypoxia, which can cause the recruitment of cells with enhanced growth, migratory and pro-mitogenic features in the wall of distal pulmonary arteries [12]. Factors that appear to drive these phenotypic changes, include platelet-derived growth factor (PDGF), and other mitogens such as the chemokine stromal cell derived factor, SDF-1/CXCL12 and the calcium binding protein and a mediator of metastasis, S100A4 [12].

2. Vascular wall remodeling in PAH

The aggressive pulmonary vascular obliterative disease characteristic of PAH, as already mentioned, involves all cell types within the vessel wall. Smooth muscle cells are initially hyperplastic and hypertrophied, and then become atrophied as fibrotic intimal proliferation develops. Adventitial fibroblasts proliferate and migrate. Endothelial damage is marked and plexiform lesions are thought to consist of proliferating abnormal endothelial cells which may sometimes consist of monoclonal endothelial cell expansion [6,13]. Thus it comes as no surprise that patients with PAH have elevated levels of several growth factors, including PDGF, VEGF, TGF-β, epidermal growth factor, fibroblast growth factor (FGF) and angiopoietin [6,7,14,15]. A number of proproliferative signalling pathways involving growth factors, cytokines, metabolic signalling, and elastases and proteases have been identified in the pathophysiology of PAH. These combine to induce proliferation and migration of smooth muscle, endothelial cells and fibroblasts, while VEGF and angiopoietin (1 & 2) are also key markers of angiogenic remodelling. Metalloproteinases

(MMPs) which are also elevated in PAH [15] and other inflammatory lung diseases [16] contribute to structural remodeling, by growth factor activation, degradation of extracellular matrix proteins (collagens, gelatins and proteoglycans) and disruption of the internal elastic lamina [6]. Inflammation is also a key component of PAH, with thrombotic lesions and infiltration of T-cells, monocytes, macrophages, dendritic and mast cells into other types of lesions, commonly observed [7,14]. While traditionally inflammation is thought to center around the recruitment of inflammatory cells to the intima of blood vessels driven by the expression of adhesion molecules on the endothelium (see later), increasing evidence suggest that inflammation may be initiated and/or driven from within the perivascular and adventitial layers - the so called "inside out hypothesis". Here adventitial fibroblasts play a key role in providing the environment in which leukocytes can rapidly infiltrate and recruit monocytes and progenitor cells to the site of vascular injury, where they produce many factors driving expression of adhesion molecules, growth factors, cytokines and chemokines [7]. Of note, the adventitial layer was reported to undergo the highest expansion in arteries between 50-100 μm in IPAH patients, with increased collagen deposition the major cause of this increase in thickness [17]. The extent to which venous remodelling contributes to the pathology in IPAH is currently a subject of much debate [7], and is thwarted by the difficulty in identifying veins that might masquerade as a remodelled artery, and for which there is no consistent cellular biomarker. That said, in a systematic study of veins identified as vessels having a single elastic lamina, a doubling of inimal and adventitial layers was documented, but reported to result from collagen deposition rather than cell proliferation [17]. Evidence of chronic inflammation with resident monocytes and intra alveolar macrophages was also seen in 65% of patients. Thus structural changes in the venous circulation probably contribute to disease pathology in PAH.

2.1 Rationale for PAH therapy: Beyond just pulmonary vasodilatation

The rationale behind the treatment of PAH is to lower pulmonary arterial pressure (PAP) and hence bring about a reduction in pulmonary vascular resistance (PVR) and right heart afterload [1]. Thus it is not surprising that therapies based around promoting vasodilatation have been developed, and include PGI_2 (epoprostenol) or stable analogues, endothelin-1 (ET-1) receptor antagonists (ERAs) and cyclic GMP (cGMP) elevating agents to increase nitric oxide (NO) bioavailability by preventing the breakdown phosphodiesterase type five (PDE5) (siledenafil, tadalafil) or that stimulate guanylyl cyclase (riociguat) [18,19]. Randomized controlled trials have described improvements in pulmonary hemodynamics, exercise tolerance and clinical symptoms with each class of agents, though to date the only monotherapy demonstrating a significant impact on long-term survival (3-5 yrs) in controlled clinical trials is PGI_2, where survival at 3 and 5 years was 63% & 55%, respectively in PAH patients [1]. In open labeled or retrospective long-term analysis of subcutaneous (SC) treprostinil, patterns of observed survival versus predicted appear to be similar or possibly slightly better (69% at 3 years) than those seen with IV epoprostenol involving patients with similar baseline characteristics [20-24]. In the latter study, this occurred despite a high proportion (44%) of patients in WHO functional class IV. Moreover, in the same study outcomes were beyond expectations in a group of patients who could tolerate up-titration of treprostinil beyond 6 months, with survival being 57% at 9 years, though overall the rate in the whole patient group at this time was 35% [24]. Comparisons with iloprost are difficult to make, given the most common formulation of this drug is inhalation. Where data exists, event free survival rates are poor (20% at 3 years) though a 59% survival rate was recorded at 3 years [25] accepting 20% of patients were on an additional therapy. It should be noted that transition from inhalation to IV late on in the disease was reported not to improve outcome [26]. In the only long-term randomized controlled trial of beraprost, this agent improved exercise capacity with evidence of less

disease progression at 6 months, though such beneficial effects were not present at >9 months [27]. Taken together, differences in the efficacy and survival rates within the PGI_2 class of drugs occurs, may reflect either their distinct pharmacology [28-30] and/or their differential ability to inhibit human pulmonary artery smooth muscle cell (PASMC) proliferation [31]. To this end it is worth noting that the rank order of PGI_2 analogues to inhibit normal human PASMC proliferation was treprostinil (UT-15) >iloprost>beraprost [31] thus at least matching clinical observations about long-term efficacy.

Despite the wide use of vasodilator therapy in PAH, pulmonary vasodilation does not necessarily predict either a fall in PVR or a positive benefit, nor is it consistently observed beyond the first 3-4 few months of therapy [32]. Indeed <15% of PAH patients are responsive to any type of vasodilator therapy at all, and of those, only ~6% respond to long-term oral calcium channel blocker therapy [33]. Likewise, significant clinical improvement exceeding that predicted from vasodilator testing alone is widely documented [1,18,34,35]. Taken together, this strongly suggests the long-term benefits of PGI_2 and its stable analogues in PAH are related to other biological properties of these agents, probably resulting from their known anti-proliferative and anti-inflammatory properties in many cell types [36,37]. This has led to the widely held view that prostacyclins slow or may even reverse the vascular remodelling process in PAH, as clearly demonstrated in experimental models, even when iloprost or treprostinil are administered after the establishment of PAH [38,39]. Indeed all 3 main class of agents used to treat PAH are expected to promote inhibition of vascular smooth muscle cell growth [40,41] with antiproliferative effects of prostacyclins, cGMP elevating agents and endothelin antagonists recognised as an important property of these agents in ACCF/AHA 2009 Expert Consensus Document on PH [1]. Few studies have however attempted to compare relevant biological readouts of current treatments. When we compared the antiproliferative properties of clinically

approved therapies under the same experimental conditions, the lowest dose to significantly inhibit ET-1 induced cell growth in PASMCs derived from PAH patients, varied 10,000 fold (0.1nM-1μM) with a rank order being treprostinil>riociguat≥selexipag metabolite≥tadalafil=iloprost>>macitentan≥bosentan [42]. Thus PGI_2 mimetics and cGMP elevating agents appear substantially more effective as antiproliferative agents than mixed (ET_A and ET_B) ERAs. This is a somewhat surprising result, given that ET-1 contributes to enhanced proliferation in PAH through increased smooth muscle receptor expression [5,43], but may reflect the fact that ET-1 binds with high affinity to ET_A and ET_B receptors with a half-life of dissociation lasting hours, making them a challenge to antagonise pharmacologically [44]. That PGI_2 and cGMP elevating agents are effective as anti-proliferative, suggests these agents can either act downstream of ET-1 receptors and/or inhibit ET-1 synthesis, as has been documented for both prostacyclins and cGMP elevating agents [5,45,46]. Thus cAMP and cGMP elevating agents given alone or in combination may be a good strategy in those patients who have high ET-1, levels of which predicts a poor survival outcome in PAH. These results therefore predict a hugely variable response on cell proliferation for current therapies.

For the remainder of this article, I will focus on the recent advances in our understanding of the mechanistic basis for PGI_2 effects on smooth muscle cell proliferation and vascular remodeling through classical (membrane IP receptors) and non-classical (nuclear receptors) pathways. In addition, I will discuss the implications arising from the key differences in the pharmacological action of PGI_2 agonists which may have an impact on their therapeutic targets and/or give rise to agents with different efficacies and side-effect profiles. I will also discuss the evidence that prostacyclins have potent anti-inflammatory properties with the ability to also to promote endothelial repair and regeneration.

3. PGI_2, classical biological target and early clinical use

PGI_2 is a 20 carbon prostanoid derivative formed within vascular endothelial and smooth muscle cells in response to the oxidation of arachidonic acid by cyclooxygenase (COX-1 and COX-2) enzymes [47]. Back in late 70s, John Vane and co-workers reported that arteries (but not platelets) contained an enzyme (PGI_2 synthase; PGI_2S) which transformed prostaglandin intermediates to an unstable substance (PGI_2) that inhibited platelet aggregation in a cyclic AMP-dependent manner (reviewed in [47,48]. The nature of the receptor pathway was confirmed two decades later when the seven-transmembrane PGI_2 (IP) receptor from human, mouse and rat was cloned and expressed in cells in 1994 by Narumiya and colleagues (see [49] for details). Aactivation of this plasma membrane receptor is coupled via Gs to adenylyl cyclase and cyclic AMP (cAMP) generation [49]. Once elevated, cAMP is rapidly broken down by specific phosphodiesterases (PDE), with PDE 1, 3, 4 largely responsible for regulating basal levels and analogue-induced elevation in the lung [50-52]. Experiments in IP receptor knockout mice confirmed that the anti-aggregatory and blood pressure lowering properties of the PGI_2 analogue, cicaprost was indeed through this receptor [53] as were the majority of its antiproliferative effects in cultured mouse PASMCs [9].

Not long after the discovery of PGI_2, this agent (epoprostenol) was given to patients with PAH and shown to be a potent dilator of both the systemic and pulmonary circulation [54], including in a subset of severely diseased patients not responding to traditional oral vasodilator therapy at all [55]. At the time of these small scale studies, much of the focus of PGI_2 in the clinical arena, was for the treatment of peripheral vascular disease such as critical limb ischemia associated with atherosclerosis and Buerger's disease [56]. Mechanistic studies in the late 80's, suggested that PGI_2 was a potent inhibitor of growth factor release from platelets and leukocytes, in particular PDGF, a key driver of smooth

muscle cell proliferation and neointimal formation in atherosclerosis [57]. This coupled with lipid lowering and anti-proliferative effects of PGI_2 in cultured aortic cells from a number of species, including human, were all evidence for the anti-atherosclerotic potential of PGI_2 (reviewed in [57]. Subsequently, the importance of PGI_2 in this disease process has been further highlighted in gene transfer and deletion experiments. Thus overexpression of PGI_2S inhibited neointimal formation in animal models of arterial injury [58,59] and vascular smooth muscle cell proliferation induced by serum [60]. In contrast, deletion of the synthase in mice resulted in raised blood pressure, fibrosis, and the development of vascular disorders with thickening of vascular walls in the kidney but also in aorta with age [61]. Likewise, in IP receptor knockout mice, greater platelet activation and vascular smooth muscle cell proliferation in response to vascular injury were observed as well as enhanced atherogenesis when either the low density lipid (LDL) -receptor or apolipoprotein-E (apoE) gene was knocked out together with the IP receptor [62,63]. Loss of the IP receptor, while not affecting blood pressure, does lead to an exaggerated hypertensive and remodeling effect of hypoxia in the lung [64], as well as an augmented cardiac hypertrophy in response to pressure overload [53,64,65] and mice develop salt-sensitive hypertension [66]. Furthermore, the potent angiogenic and anti-remodelling effects of bone-marrow derived endothelial progenitor cells (EPCs) in both a hind limb ischemia and vascular injury model were impaired in $IP^{-/-}$ null mice [67,68]. The mechanism may in part be due a reduction in adhesion of EPCs to the relevant sites of repair [68].

Thus, there is strong evidence that PGI_2, through activation of its classical biological target, the IP receptor, is protective in the context of proliferative diseases such as atherosclerosis and PAH.

4. PGI_2 analogues signal through multiple prostanoid receptors

Prostacyclin is clinically hard to work because it is chemically unstable with a half-life either *in vitro* or *in vivo* of approximately 3 minutes at a physiological pH and temperature [69]. Given this inherent instability, chemists set about making a series of compounds based around PGI_2 that were not susceptible to hydrolysis in solution and had a longer biological half life. Iloprost, which shares the same pentanoic side chain to PGI_2, will however very slowly degrade at room temperature and is most susceptible to β-oxidation making its terminal plasma half-life less (20-30 mins) than either beraprost (40-60 mins) or treprostinil (180-270 mins) [70-72]. Several drug formulations now exist for clinical use including intravenous (iloprost, treprostinil), oral (beraprost, treprostinil), inhaled (iloprost and treprostinil) and subcutaneous (treprostinil). As shown in table 1, like prostacyclin, all stable PGI_2 analogues potently bind to the IP receptor. Iloprost is approximately 10 fold more potent than treprostinil [29], consistent with the observed potency difference of these two agents to inhibit IP-dependent relaxation in human pulmonary artery [73]. Selexipag (NS-304) is a novel non-prostanoid moiety that is currently being developed as an oral drug for PAH [74], with Phase III trials (GRIPON) now complete. It differs from other PGI_2 analogues in that selexipag is a pro-drug with a relatively low affinity at the IP receptor (Ki 260 nM), but is rapidly hydrolyzed *in vivo* to an active metabolite, MRE-269 (ACT-333679), that has a high affinity for the human IP receptor (Ki 20 nM) and a long (~8hr) plasma half-life [75,76]. Despite the selexipag metabolite having a high IP receptor potency, it like PGI_2, was comparatively weak at relaxing in human pulmonary artery, with its concentration-response curve shifted by two orders of magnitude to the right compared to iloprost [73]. The reason for this discrepancy is not immediately obvious considering that neither selexipag nor its metabolite have significant binding affinity ($Ki \geq 2.6\mu M$) for any other prostanoid receptor that might oppose its action at the IP receptor, but might be explained on the basis of multiple targets for the other analogues (see below). In other

experiments where IP agonists have been compared as antiproliferative agents under the same experimental conditions, effects of iloprost and MRE-269 were broadly similar, whereas treprostinil was an order of magnitude more effective [42], as observed previously when analogues were compared in normal human PASMCs [31].

Increasing evidence suggest that prostacyclins have significant actions at other prostaglandin receptors which might contribute to or modulate their therapeutic action (figure 1). The EP_2, EP_4 and DP_1 receptors are vasorelaxant receptors coupled to Gs and therefore elevate intracellular cyclic AMP levels while EP_1, EP_3, FP and TP are contractile receptors coupled to Gi and Gq that either elevate intracellular Ca^{2+} and/or reduce cyclic AMP [77]. Although difficult to assess in binding studies, PGI_2 is known to have poor selectivity for prostanoid receptors, binding to and activating EP_1, EP_3 and TP receptors, albeit at higher concentrations (15-45 fold for EP_1 and EP_3 and <100 fold for TP) compared with the corresponding natural ligand [78-80]. This still means that such receptors will be significantly activated by PGI_2 between 10-40 nM (EP_3) and ~100nM (EP_1 & TP), with the plasma concentration of epoprostenol (as measured by its metabolite, 6-keto-prostaglandin $F_{1\alpha}$) in PAH patients estimated to be ~25nM [81]. Activation of these receptors would therefore lead to vasoconstriction, thrombosis and cell proliferation [49,82]. In human lung and platelets, EP_3 and TP receptors are the main prostanoid receptor to oppose signalling through IP receptors, the exception being pulmonary veins where EP_1 and TP receptors appear to counteract prostanoid-induced relaxation [82,83]. Thus PGI_2 has the potential for deleterious effects if IP receptor expression is compromised and/or if TP/EP receptor signaling is enhanced. Indeed, in mice genetically deficient in the IP receptor, vascular proliferation and platelet activation are increased in response to tissue injury whereas the opposite occurs in TP receptor knockout mice, [84]. Moreover, thromboxane levels rise when the IP receptor gene is deleted and the vasodilator response to cicaprost is converted

to vasoconstriction [84], presumed to relate to activation of the EP_3 receptor which is negatively coupled to adenylyl cyclase via Gi [85,86]. Taken together, this suggests that the balance between these receptor pathways is crucial in maintaining vascular homoeostasis. The PGI_2 and thromboxane pathways are intrinsically linked at the receptor level since the TP receptor can dimmerise with the IP receptor in native cells, where TP agonists behave in an "IP-like" manner, and actually increase intracellular cyclic AMP. Such an association, serving to limit the deleterious effects of thromboxane, was reported recently to be disrupted under conditions of hypercholesterolemia [87]. Given that IP but not TP homodimer function was shown in the same study to be down-regulated, suggests that atherosclerotic disease preferentially enhances TP receptor signalling. This may in part explain why, apoE null mice fed a high cholesterol diet, could develop PAH [88].

Like PGI_2, iloprost has poor selectivity for prostanoid receptors, being essentially equipotent at activating both IP and EP_1 receptors [49,89], and this was recently confirmed [29]. Thus it is not surprising that iloprost-induced vasorelaxation can be enhanced by EP_1 receptor blockade in the isolated rabbit perfused lungs [52] or in guinea-pig aorta [90]. The significance of EP_1 receptor activation is not widely understood, but its activation may contribute to loss of efficacy clinically in PAH, either through enhanced IP receptor desensitisation [52] or reduced cAMP generation through activation of the calcium/calmodulin-dependent PDE1 isoform [91]. Functional EP_1 receptors are found in the gastrointestinal track, causing contraction of the human colon, while in the stomach, these receptors can have either detrimental or beneficial effects, worsening histamine-induced gastric injury and delaying gastric emptying while providing cytoprotection against acid- and ethanol- induced injury of the mucosa [77,92].

Iloprost has been reported to have significant activity at the human EP_4 receptor, though not at the mouse receptor (Table 1). Despite notable expression of EP_4 receptors in the human lung [93], they do not appear to mediate relaxation of human pulmonary arteries but may in veins [82,83], though relaxations induced by iloprost (and treprostinil) were recently found to be insensitive to EP_4 antagonists in both arteries or veins [73]. In situations where the IP receptor is down regulated (in PAH or high cell passage number), it is possible that EP_4 receptors may generate sufficient cyclic AMP for a functional response on vascular tone or cell proliferation [93]. However, this would appear to occur at concentrations well outside (>50 fold) the therapeutic dose range [29,89], with the upper plasma concentration achieved in patients with this drug being close to 1 nM [94]. This strongly suggests that EP_4 receptors are not likely to be a clinically relevant target for iloprost.

Until recently, little was known about the pharmacology of treprostinil, though enhanced and more prolonged cAMP generation compared to other analogues was originally reported in both human PASMCs and mouse alveolar macrophages, strongly suggesting signalling through additional Gs coupled receptors [31,95]. This was largely accounted for in macrophages by activation of EP_2 but not EP_4 receptors [95]. In comparative binding studies, treprostinil, in contrast to iloprost, was shown to have high affinity for the DP_1 and EP_2 receptor (Ki 4.4 & 3.6 nM, respectively) while having a 100 fold lower affinity for EP_1 ([29] and Table 1). A similar pattern was observed for functional assays (cyclic AMP and calcium) in the same study, confirming the unique pharmacology of treprostinil with respect to iloprost and other PGI_2 analogues [89]. Consistent with these findings, treprostinil was a more potent vasodilator of human pulmonary veins than arteries and DP_1 receptor activation could account for this, whereas PGI_2 was a poor venorelaxant, while iloprost was equipoent [73]. Moreover, when treprostinil was assessed as a vasorelaxant in several isolated smooth muscle preparations, it was equipotent with PGE_2 against EP_2

receptors (EC_{50} 4-5 nM) in mouse trachea and only 3–4 times less potent than PGD_2 at DP_1 receptors in rabbit saphenous vein and vena cava [96]. Thus in functional assays, treprostinil is behaving like potent EP_2 and DP_1 agonist. Given the plasma concentration achieved with intravenous or subcutaneous treprostinil in patients ranges between 2.5 to 25 nM [97], strongly suggests EP_2 and DP_1 receptors will be activated within the therapeutic window of this drug. Thus activation of IP, DP_1 and EP_2 receptors, all of which are linked to cyclic AMP generation could act in concert to produce the biological effects of treprostinil.

One consequence of having an agent that can potently relax veins might be to prevent pulmonary oedema, commonly caused by vasoconstriction or loss of vein distensibility by collagen deposition [98]. Both endothelin and thromboxane constrict human veins more potently than arteries and increase microvascular pressure, suggesting when their levels are high, this will increase the risk of oedema in PAH (see [98] for discussion). PGI_2 has detrimental effects in patients with pulmonary veno-occlusive disease (POVD), presumably because it will cause pooling of the blood in the veins due to a mismatch of arterial and venous blood flow, whereas this is not likely to happen with iloprost and treprostinil [73]. Thus one might argue in favour of either agent, particularly treprostinil, being considered in post-capillary disease, where venous remodelling due to high pressure is a major problem. Another prediction from the pharmacology of treprostinil is that it will be a particularly effective bronchial dilator and inhibitor of platelet function as human bronchial smooth muscle [99,100] and platelets [101,102] both express functional IP, DP_1 and EP_2 receptors. Indeed treprostinil was reported to inhibit human platelet aggregation with an EC_{50} in the subnanomolar range [103]. EP_2 receptors have other important functions to consider in the context of a fibrotic disease like PAH. These receptors significantly contribute to the antiproliferative effects of PGE_2 in cultured human airway smooth muscle and fibroblasts

[104,105]. The role of EP_2 receptor in the context of remodelling in PAH is unknown, though neointimal hyperplasia in response to injury was accelerated in $EP2^{-/-}$ mice, and associated with increased proliferation and migration of smooth muscle cells [106]. It is also important to note that activation of EP_2 receptors have a range of inhibitory effects on fibroblast function (proliferation, migration, transition from fibroblast to myofibroblast) that is driven largely by PDGF and TGFβ, which could therefore contribute to the beneficial effects of treprostinil in PAH [107-109].

Other potential targets for PGI_2 and its analogues include EP_3 receptors [89] which may negatively modulate vasorelaxation induced by analogues and [76,86,110], particularly in small pulmonary vessels where EP_3 agonists are more effective vasoconstrictor agents [110]. Extrapolation from historical dose-ratios comparing the functional effects of prostaglandin E_2 and PGI_2 with the binding data in table 1, would make PGI_2, the only prostacyclin drug to potentially activate EP_3 receptors in the clinical dose-range. Indeed, in the rat fundus potent (EC_{50} 53nM) contractile effects are reported for PGI_2 [80], with double that required to elicit contractions with iloprost in the same preparation and even higher (1 and 10 μM) for beraprost and treprostinil, respectively [111]. The situation may differ under conditions when IP receptors are down regulated as occurs in PAH [9,93]. Furthermore, contractile agents, particularly phenylephrine, can markedly potentiate the effects of EP_3 agonists, where strong contractions are only observed with some 'priming' beforehand [112] which makes PGI_2 to activate TP receptors in the sub nanomolar range and contract rat pulmonary arteries further [113]. This situation may be relevant under conditions of high sympathetic tone coupled to high thromboxane levels as is likely to occur in PAH.

5. PGI_2 signalling through nuclear receptors

Apart from clear differences in the functional consequence of gene deletion of the IP receptor and PGI_2S, it has been acknowledge for a while that IP receptors alone cannot fully account for the known biological actions of PGI_2. Since the discovery that PGI_2S has a bimodal distribution in vascular smooth muscle cells, showing strong expression in both plasma membrane and perinuclear regions [114], the importance of PGI_2 signalling through a family of transcription factors called peroxisome proliferator-activated receptors (PPARs) has now been widely recognised [115,116]. PPARs are generally activated by ligand binding and contain a central DNA-binding domain that recognises response elements in the promoters of their target genes. Three main isoforms exist, PPARα, PPARβ/(δ) and PPARγ, and in the mid to late 90's were shown to be activated by a variety of endogenous ligands, including prostaglandins (e.g. prostaglandin A_1, A_2, B_2, D_2, 15-Deoxy-$\Delta^{12,14}$-PGJ_2,), fatty acids (e.g. linoleic), and lipoxygenase metabolites (*e.g.* 8-HETE) as well as a variety of synthetic agents [117,118]. At the time of these experiments, it was noted that the PGI_2 analogues, iloprost and carbacyclin could directly bind to PPAR α or β and activate them as efficiently as endogenous and synthetic ligands [118]. This was not a property shared by cicaprost, and for that matter PGI_2, though with the latter, this was presumed to be due to its rapid hydrolysis under experimental conditions [118]. Using crystal structures of the ligand binding domain, the structural basis for iloprost binding to PPAR α & β has now unequivocally been confirmed, though interestingly direct binding to PPARγ with iloprost could not be demonstrated [119]. This distinctive property of iloprost and of other analogues, including beraprost [120] and treprostinil [103], has often been used to distinguish between signalling through the IP receptor and PPARs. There are a number of instances where the angiogenic, anti-tumour or antiproliferative effects attributed to PGI_2 or analogue activation of PPARs is either not mimicked by cicaprost and/or does not involve IP receptors [9,121-123]. Nonetheless, there are circumstances where PPAR activation can

be driven by the IP receptor and/or be seen with cicaprost [124,125], suggesting cross talk between PPARs and IP receptors does actually occur. Indeed cyclic AMP-dependent protein kinase A (PKA) is known to phosphorylate and activate the ligand binding domain of PPARs [126]. However, IP receptor-dependent activation of PPARγ, while involving phosphorylation of the ligand binding domain, was found to be independent of cyclic AMP generation and PKA [125]. Given that many other kinases can regulate PPAR activity, including GMP-dependent kinase both in the absence and presence of ligands [127,128] suggests complex regulation by other receptors and/or signalling pathways.

Thus signalling through PPARs represents an important biological target for prostacyclins. Indeed, neither the IP receptor nor cAMP appears to be involved in meditating iloprost relaxation in some rodent blood vessels [30,129] possibly suggesting a major role for PPARs. However, a lot more work is required to tease out the specific role of membrane receptor activation versus direct analogue binding, the cross talk between the two, as well as signalling through endogenously produced ecosanoids, which so far has largely been ignored. The possibility that both membrane and nuclear signalling are required to be inhibited before the role of either pathway can be confirmed should also be considered (c.f. [96]).

The cellular function of PPARs is diverse, regulating processes such as lipid and glucose metabolism, insulin sensitivity, cell growth, apoptosis and inflammation [115,127,130]. Synthetic and highly selective activators exist for each of the PPAR isoforms and these have been extremely useful in identifying the physiological and pathophysiological function of these transcription factors in different cell types. Fibrates activate PPARα and are used clinically in the treatment of hyperlipidaemia while glitazones activate PPARγ and are used to treat type-2 diabetes [127]. GW501516 and GW0742 are highly selective

activators of PPARβ and their development has mostly been directed towards treatment of metabolic syndromes [116].

5.1 Vascular protective effects of PPARs

All three PPAR isoforms are highly expressed in endothelial cells and to varying degrees in vascular smooth muscle, with PPARβ having the most widespread distribution [127] and probably the highest expression in pulmonary vessels [131,132]. Given that ligands of all three isoforms can promote vasorelaxation [133,134], including in the lung [131,135,136], strongly suggests that PPARs are likely to contribute to the blood pressure lowering effects of PGI_2 [61]. Perhaps in the case of PGI_2 itself, the mechanism occurs independent of the IP receptor, given $IP^{-/-}$ null mice are normotenstive [53,64] but PPAR activation is likely to involve an increase in endothelial production of NO through phosphatidylinositide 3-kinase-dependent eNOS phosphorylation and a reduction in ET-1 1 levels [133,134,137,138]. So far, the only PPAR isoform reported to significantly contribute to the vasorelaxing effect of PGI_2 analogues, is PPARβ in pulmonary arterial smooth muscle [131,139]. In the former study, evidence was provided that PPARβ/δ mediated effects involved activation of the large conductance calcium-activated potassium channel in an IP receptor- and PKA- dependent but nitric oxide-independent manner in human PASMCs. In rat pulmonary artery, the IP receptor probably mediates treprostinil and beraprost induced activation of PPARβ [30,139].

Several studies have also shown increased vascular endothelial cell growth factor (VEGF) production following treatment with PGI_2, iloprost, beraprost and treprostinil in many cell types both *in vivo* and *in vitro* [15,140,141]. This appears to be linked to cyclic AMP and activation of the cAMP-responsive element binding protein (CREB) [142,143] but also reported to involve PPARα [141] and PPARβ [144,145]. VEGF is linked to increased

production of NO and PGI_2 in endothelial cells [146] and the stimulation of EPC proliferation to promote neovascularisation [147] or the formation of new blood vessels [141,144]. Thus PGI_2 activation of PPARα and PPARβ may have a key role in maintaining endothelial integrity and regeneration. Indeed both iloprost and treprostinil can increase the number and angiogenic potential of EPCs either in patients with PAH [148] or critical limb ischemia [149] and in an *in vitro* assay of endothelial tube formation [123]. In the latter, COX-1, PGI_2S and PPARβ expression was strong in these human EPCs, and all critical for *in vivo* capillary formation when these specialised cells were transferred to nude mice [123]. Furthermore, the importance of VEGF in the context of maintaining vascular homeostasis in the lung is highlighted by studies showing that VEGF receptor blockade causes mild pulmonary hypertension (PH) and muscularisation of pulmonary arteries associated with endothelial cell death, which when combined with chronic hypoxia, animals present with severe PAH and occlusive vascular lesions [13]. It is likely these regenerative mechanisms underpin the clinical benefit of these PGI_2 analogues in peripheral vascular disease as well as Raynaud's phenomenon and digital ulcers associated with scleroderma [150,151]. Although cicaprost advanced into clinical trials, treatment of vasospasm in scleroderma patients was shown to be efficacious with iloprost but not with low-dose oral cicaprost [152], despite the latter having potent anti-platelet and blood-pressure effects in human volunteers [153]. Thus with the benefit of hindsight, perhaps lack of efficacy relates not only to the inability of cicaprost to bind and activate PPARα/β but also its inability to enhance VEGF expression and drive angiogenesis in vivo [121]. This is in direct contrast to iloprost.

PPARγ in conjunction with the other PPARs is also likely to impact on endothelial cell function. Agonists of PPARγ cause endothelial tube formation in an endothelial/interstitial cell co-culture assay as well as neovascularization *in vivo*, via an effect associated with

increased production of VEGF [154]. Its knockdown in endothelial cells leads to an abnormal, proliferating, apoptosis-resistant phenotype [155], and mice develop spontaneous PAH [156]. PPARγ expression in this cell type is reported to be down-regulated in PAH patients [9,155], suggesting that its loss may strongly impair endothelial cell function. The BMP receptor type 2 (BMPR2), loss of function mutations of which underlie the majority of heritable PAH, is known to be critical for maintaining endothelial cell homeostasis [7]. In work carried by Rabinovitch and colleagues, a BMPR2-mediated complex between PPARγ and β-catenin was found to be critical for promoting pulmonary artery endothelial cell survival, proliferation, and migration via its transcriptional upregulation of the apelin gene (reviewed in [6]). This and other work performed in the same laboratory, has provided compelling evidence that PPARγ is a critical downstream target for BMPR2 in both endothelial and smooth muscle cells.

PPARs can also play a role in the maintenance of a smooth muscle contractile cell phenotype. Thus shear stress applied to endothelial cells co-cultured with smooth muscle cells resulted in increased smooth muscle cell PPAR α, β, γ ligand activity and increased contractile markers together with decreases in proinflammatory gene expression (MCP-1 and interleukin-8). However, knockdown of either endothelial PGI_2S or PPARs confirmed a role for PPARα nd β but not PPARγ [157]. In other studies, PPARγ, appears to reduce neointimal hyperplasia by maintaining a smooth muscle contractile phenotype via either up-regulation of protein kinase G [158] and/or adiponectin [159].

To summarise, the data discussed above supports the increasing body of evidence that PGI_2 signalling through PPARs is part of a previously unrecognized mechanism that contributes to the vasoprotective and regenerative effects of PGI_2 on the endothelium, a subject which

has also been extensively reviewed, mainly in the context of atherosclerosis [116,127] but to also maintaining a smooth muscle contractile phenotype.

5.2. IP receptor and PPARs as regulators of cell proliferation

Previous studies in normal human PASMCs have shown that PGI_2 analogues can inhibit the mitogenic responses to PDGF and serum in a largely cAMP-dependent manner, with adenylyl cyclase inhibitors blocking around 75% of the analogue responses in these cells [31,160]. Likewise, the growth rate of HEK-293 cells stably expressing the human IP receptor (HEK-IP) was substantially slower compared to cells expressing only the empty vector, and treprostinil inhibited cell growth in HEK-IP cells in a largely but not exclusively cAMP-dependent manner but one that also involved PPARγ [9,125]. Cyclic AMP-dependent activation of protein kinase A (PKA) has been described to underlie PGI_2 analogue-dependent effects on cell proliferation induced by either serum [39], PDGR [39,161] and TGR-β1 [11], though in most studies, H-89 has been used which is known to block many kinases beside PKA [125].

The downstream mechanisms of how cyclic AMP might inhibit growth are not well understood, but PGI_2 analogues appear to inhibit smooth muscle cell proliferation by blocking progression from G1 to S phase [161-163]. This may occur through phosphorylation of the cAMP response element binding protein (CREB) [39,162,164,165] with downstream targets including the inducible cAMP early repressor gene [161], cyclin A [162] and/or p21/p27 [164].

There are other mechanisms by which cAMP cascades can impede cell growth. PGI_2 analogues can suppress TGF-β signalling either through inhibiting phosphorylation of SMAD 2/3 [11,165] and through SMAD independent dephosphorylation of p38 MAPK

[11] and can up-regulate BMPRII signalling through enhanced BMP phosphorylation of SMAD 1/5 and expression of inhibitory DNA biding protein 1 (Id1), the latter occurring irrespective of BMP ligand activation and SMAD phosphorylation [39]. In other studies, treprostinil inhibits serum-induced cell growth in human PASMCs in part through cAMP-dependent activation of ATP-sensitive potassium K^+ channels [166]. Recently mutations in TASK-1, a background potassium current switched off by hypoxia and ET-1 in human PASMCs, gives rise to a rare form of heritable PAH [167]. PGI_2 analogues can activate this current, and possibly rescue trafficking defects in TASK-1, but the role of this current in driving pulmonary cell proliferation and remodelling remains to be determined. In aortic smooth muscle cells, cAMP elevating agents inhibit PDGF-induced proliferation through inhibition of the calcineurin and NFAT (nuclear factor of activated T-cells) pathway [168]. The exact mechanism was not fully explored, but PKA is known to promote the nuclear export of the calcineurin/NFAT complex while a lowering of intracellular calcium levels would inhibit the phosphatase activity of calcineurin thereby maintaining NFAT in the phosphorylated state and preventing its translocation into the nucleus (see[4,168]). Such a mechanism may counteract the elevated calcineurin/NFAT activity and expression reported in IPAH or in remodeled pulmonary arteries [169]. Furthermore, this transcription factor appears largely responsible for the down-regulation of voltage-gated (Kv) current and Kv1.5 expression reported in human PASMCs from these patients as well as the expression of pro-inflammatory cytokines and growth-promoting genes (including ET-1) in a number of different cell types [4,170]. PGI_2 analogues have been reported to reduce ET-1 synthesis stimulated by mitogens in PASMCs [45,171] and to reduced elevated plasma levels in patients with systemic sclerosis [172]. Whether this relates to inhibition of NFAT activity, which itself can be suppressed by a direct interaction of PPARγ [16], remains to be determined, but likely to play a significant role. Moreover, PPARγ negatively regulates

store-operated Ca^{2+}entry (SOCE) through the down regulation of TRPC1 and TRPC6 [128], which may impact indirectly on calcineurin/NFAT signalling.

Activation of PPARs is also likely to contribute to the antiproliferative effects of prostacyclin drugs in a variety of cell types. Recent studies have shown that PPARγ contributes in part to antiproliferative effects to PGI_2 analogues in normal PASMCs [9] and interestingly also to the PDE5 inhibitor, sildenafil [128]. In other studies, iloprost-induced inhibition of lung tumourigenesis was shown to be mediated by PPARγ, where pulmonary-specific over expression of either PGI_2S or PPARγ resulted in suppression of tumour incidence and multiplicity in these lung models while gene deletion of the IP receptor had no effect on tumour growth[122]. Furthermore non-small cell lung cancer cells over-expressing PPARγ exhibit significantly less invasiveness and metastases [122]. Taken together this suggests a key role for both PGI_2 and PPARγ in regulating cancer progression. A contribution from PPARβ was ruled out on the basis that iloprost could not activate a PPARβ-specific response element in lung epithelial cells [122]. This contrasts with other studies where PPARβ appears to mediate the antiproliferative effects of treprostinil in human lung fibroblasts [173] or beraprost in cultured aortic smooth muscle [120,164]. A consistent finding with many of the studies described above, is that PPAR activation by analogues occurs at higher concentrations than those required to activate IP receptors. This would fit in with the observation that iloprost binding to the crystalised ligand-binding domain of PPARα or β occurs with an EC_{50} of ~200nM [119]. Accepting the limitations of the somewhat artificial nature of this study, suggests some PPAR effects could lie outside the therapeutic concentration range or require up-titration of the prostacyclin drug beyond the initial hemodynamic response.

The role of PPARβ in the cardiovascular system is complex, since over expression of PPARβ actually promotes vascular smooth muscle proliferation and atherosclerosis [127] and has tumorigenic effects [116]. Furthermore, PPARβ activation is consistently associated with human lung carcinoma cell growth (e.g. [174]). In contrast, PPARβ ligands appear to do the opposite and inhibit PDGF-mediated effects on human PASMCs proliferation, and migration [132]. A degree of caution must however exercised when interpreting data using PPARβ ligand activators in isolation, as the vasorelaxing effect of GW0742 in pulmonary and mesenteric artery was not altered in PPARβ null mice [175], suggesting PPARβ independent effects of some ligand activators.

6. Impact of pulmonary hypertension on PGI_2 signalling

Several studies point to multiple defects in PGI_2 signaling pathway in PAH which may either contribute to disease pathology and/or explain why PGI_2 therapy wanes as the disease progresses necessitating doses to be escalated. Decreased urinary levels of the PGI_2 metabolite, 2,3-dinor-6-keto-$PGF_{1\alpha}$ are found in patients with IPAH [176] and pulmonary hypertension (PH) linked with congenital heart disease [177,178]. In IPAH this is associated with a progressive loss of PGI_2S expression from large to small pulmonary arterial vessels, with virtually no expression in plexiform lesions [179]. In other forms of PH, PGI_2S protein is reduced in a fetal lamb model of persistent PH of the newborn, where synthase activity was also found to be decreased by protein tyrosine nitration [180]. In the context of PAH, PGI_2S is protective as overexpression in monocrotaline- and hypoxic-induced models of PAH in rodents, reduced both medial thickening and pulmonary pressure [181,182] while in a bleomycin model of acute lung injury, pulmonary fibrosis, lung inflammation and mortality were attenuated [183]. Moreover, individuals from families with heritable PAH, that had a bias for PGI_2S promoter polymorphisms with increased transcriptional activity, had a lower risk of developing PAH [184], demonstrating a

protective effect of the synthase in humans. With respect to the IP receptor, a reduction in message and protein levels have been reported in IPAH lungs or in rats following monocrotaline treatment [9,93], although in the latter study, down regulation was reported to be driven by drug treatment itself rather than the disease, but not in intimal proliferating cells, where expression was weak regardless. IP receptor loss is however unlikely to cause PAH as mice lacking the IP receptor gene do not spontaneously develop PH, although they are more susceptible to the hypertensive and remodeling effects of hypoxia [64]. Polymorphisms in the IP receptor have been associated to platelet disorders and to accelerated atherosclerotic disease in humans [185], but so far have not been directly linked to PAH. Thus there is good evidence that the dysfunction of both PGI_2S and the IP receptor is likely to contribute to the pathology and disease severity in PAH.

IP receptor signalling may also be hampered by increases in the expression and activity of PDEs. Phosphodiesterases comprise a family of at least 11 isoforms (PDE1-PDE11) that each have a different capacity for hydrolyzing cAMP, cGMP, or both [186]. Elevated message and protein levels of PDE1 (PDE1A & PDE1C), PDE5 and to a lesser extent PDE3 (PDE3A & PDE3B) along with heightened activity of these PDEs has been reported in IPAH [50,51,91]. Taken together, this suggests that reduced effectiveness of PGI_2 and its analogues due to enhanced cAMP breakdown is likely to occur in PAH. Indeed, blockade of PDE1 and PDE3 restored the ability of beraprost to increase cAMP levels in PASMCs derived from IPAH patients [51]. Furthermore, targeted knockdown of PDE1C with siRNA enhanced cAMP accumulation and inhibited cell growth more in human PASMCs from PAH patients than from controls [51]. In other studies, aortic smooth muscles cells from $PDE3A^{-/-}$ mice were less responsive to PDGF compared to cells from wild type or $PDE3B^{-/-}$ mice, in part due to PKA-mediated CREB phosphorylation, higher levels of p53 and reduced ERK-1/2 phosphorylation [187]. Thus a key role of PDE1 & PDE3 in modulating

cAMP levels growth and responses to PGI_2 analogues would fit with the observation that only a combination of a PDE1 or PDE3 inhibitor with iloprost could reverse PH and distal pulmonary artery muscularization induced by chronic hypoxia [50]. Likewise, in a chronic monocrotaline model, combined administration of iloprost and a mixed PDE3/4 PDE inhibitor was required to fully normalize hemodynamic and right heart changes when administered after full establishment of PH [38]. Taken together, these findings indicate that upregulation of PDE1C and PDE3 play a role in the structural remodeling process underpinning PAH and suggests that a PDE1 inhibitor and/or a PDE3 inhibitor might be useful in potentiating analogue effects in the clinical setting. This is probably happening to an extent when prostacyclins are combined with the PDE5 inhibitors, as PDE3-mediated hydrolysis of cAMP should theoretically be inhibited by cGMP and thus augment cAMP levels in response to prostacyclins, as recently described for treprostinil and tadalafil [188]. Sildenafil in particular is likely to inhibit PDE1 activity at therapeutic doses [186] either directly (K_i for inhibition ~280 nM) or indirectly through reducing intracellular calcium and thereby the calmodulin interaction with PDE1 [51]. The latter is almost certainly likely to be enhanced when combining with prostacyclins. Taken together, this provides a scientific rationale for the increasing number of reports showing improved hemodynamics, lengthened time to clinical worsening, and increased survival when PDE5 inhibitors are combined with prostacyclins (see[186,188]). Very recently, milrinone (PDE3 and PDE4 inhibitor) combined with inhaled PGI_2 has been assessed retrospectively in 60 high risk patients having PH after coronary bypass surgery and was shown to reduce PAP without an fall in systemic blood pressure, increase cardiac index and reduce vasoconstrictor support [189]. While there are clearly limitations with the study, such an approach warrants further investigation clinically. Cilostazol, a more selective PDE3 inhibitor, which is currently in clinical use for intermittent claudication [190], might also be considered in combination with prostacyclins.

Recently other dual substrate PDEs isoforms, which may be targeted clinically or be involved in disease progression in PAH, have recently been documented [191,192]. Strong immunoreactivity for PDE10A compared to weak expression in control lungs was observed predominantly in the medial layer of pulmonary arteries in both the lungs of IPAH patients and in rats following monocrotaline, while siRNA knockdown in PASMCs reduced proliferation by around 40% [191]. In very recent studies, a highly selective and potent PDE2 inhibitor, BAY 60-7550 elicited pulmonary dilation, prevented pulmonary vascular remodeling, and reduced right ventricular hypertrophy [192]. In addition this PDE2 inhibitor potentiated the effects of treprostinil, an NO donor, sildenafil or atrial natriuretic peptide (in neutral endopeptidase inhibitor) in established PAH. Thus dual selective PDE inhibitor may collectively enhance NO and cAMP signalling and may be a novel approach in PAH.

For a number of reasons, IP receptor desensitization is likely to be a problem with the long-term use of PGI_2. Analogues are known to induce rapid time- and concentration-dependent phosphorylation and internalization of the IP receptor [193] with desensitisation lasting for several hours after agonist removal [194]. The mechanism of receptor desensitization is mutifactorial and involves both PKA and protein kinase C (PKC). Cross-desensitization can also occur via EP_1 [52] or thromboxane A_2 (TP) receptors [195], being activated either by PGI_2 agonists themselves or by increased production of their respective endogenous ligands, PGE_2 or thromboxane A_2, both of which can increase in lung disease [176,196]. Thus in conjunction with high PDE activity, receptor desensitization, may contribute to the lack of responsiveness of PGI_2 agents in advanced disease. Interestingly, EP_2 receptors, which will be a major target for treprostinil, do not readily undergo receptor desensitisation

in human airway smooth muscle or in COS cells over expressing these receptors both under conditions where other Gs coupled receptors do [197].

The extent to which IPAH impacts on PPAR isoform expression is relatively unexplored. Over 10 years ago, Ameshima and colleagues reported reduced staining of PPARγ in IPAH lungs [155] as was recently reported in lung tissue samples and in airway epithelial cells of patients with severe chronic obstructive pulmonary disease [198]. Likewise, PPARγ expression was significantly reduced in the lungs of rodents with hypoxia induced PH [128,135,199] and also in the vascular lesions in a rat model of severe PAH caused by hypoxia in the presence of a VEGF blocker [155]. In endothelial cells and in the proliferating cells within the intima and plexiform lesions PPARγ expression was non-existent [155], a finding subsequently confirmed in our laboratory [9]. Moreover PPARγ knockdown in endothelial cells leads to an abnormal, proliferating, apoptosis-resistant phenotype [155] and *in vivo* PAH and muscularization of distal pulmonary arteries occurs [156]. Given that PPARγ, along with the IP receptor and PGI_2S are all minimally expressed in intimal proliferating cells, suggests that loss/lack of all three targets may be significantly contributing to hyperproliferation as well as influencing disease severity. However, while targeted deletion of PPARγ in smooth muscle is also known to cause PAH in mice [200], we actually found its expression to be markedly up-regulated in the medial layer of distal pulmonary arteries taken from the lungs of children with endstage PAH disease, and in contrast to the IP receptor, expression was unaffected by drug treatment [9]. Furthermore, we showed that human PASMCs from PAH patients were sensitive to the growth suppressing effects of PPARγ activator, rosiglitazone, which potentiated the antiproliferative effects of treprostinil [9]. Thus PPARγ upregulation in smooth muscle may actually serve as a compensatory mechanism to limit vascular remodeling in PAH. Consistent with this notion, PPARγ expression was reported to be increased in the smooth

muscle layers from asthmatic patients [201] as well as in atherosclerotic lesions [127]. The factors regulating PPARγ expression in PAH are not well defined, although the pro-inflammatory cytokine, IL-4 appears to be intrinsically linked to the induction of a cluster of genes associated with the PPARγ pathway, including the gene itself and three enzymes ALOX15 (arachidonate 15-lipoxygenase), MAOA (monoamine oxidase) and ENPP2 (ectonucleotide pyrophosphatase/phosphodiesterase family member 2), whose activity can potentially generate endogenous PPARγ ligands, namely activators like, 13-hydroxyoctadienoic acid (13-HODE), 5-hydroxyeicosatetraenoic acid (15-HETE) and lysophosphatidic acid [202]. In PAH, it is possible that in patients treated sildenafil, may have enhanced PPARγ expression, as cGMP/PKG is linked to its upregulation in chronic hypoxia in rats [128].

Currently there is no information about what happens to PPAR α and β in PAH, although PDGF was found to increase PPARβ expression and protein levels by 4-fold in human PASMCs [132], suggesting its expression may be unregulated in PAH, at least in smooth muscle. PPARα will suppress hypoxia-inducible factor α and ET-1 levels [203], so its loss may exacerbate the effects of hypoxia.

7. Role of PPARs in lung remodelling in the context of PAH

Several lines of evidence suggests PPARs are also targets in lung disease where proliferation, remodelling and inflammation are key components [16,130,137,204]. Iloprost or pulmonary-specific over expression of either PGI_2S or PPARγ was reported to result in suppression of tumour incidence and multiplicity in lung models [122]. In contrast, gene deletion of the IP receptor had no effect on tumour growth nor was the response to iloprost inhibited in these mice [122]. Furthermore, non-small cell lung cancer cells over-expressing PPARγ exhibited significantly less invasiveness and metastases. As already mentioned, the

role of PPARγ in vascular remodelling in the lung is strongly implicated as targeted deletion of PPARγ in either smooth muscle or endothelial cells causes PAH and muscularization of distal pulmonary arteries [156,200]. In contrast, PPARγ ligands (e.g. rosiglitazone or nitro–fatty acids) enhance the antiproliferative effects of treprostinil in human PASMCs from PAH patients [9] and protect against the pulmonary effects of monocrotaline and hypoxia in a number of PAH animal models [6,135,137]. In addition, PPARγ ligands can protect against neointimal hyperplasia induced by vascular injury [158] or a proinflammatory phenotype induced in epithelial cells by cigarette smoke [198]. Key mechanisms by which PPARγ attenuates remodelling in PAH appear to involve a dampening of PDGF receptor β (PDGFRβ) signalling through receptor internalisation and reduced ERK1/2 phosphorylation and a rescue of BMP receptor type II (BMPRII) dysfunction [6]. Likewise, beraprost was not only able to inhibit the proliferation of PASMCs expressing a mutant BMPRII receptor identified in PAH patients more effectively than wild-type cells, but it could reverse aberrant proliferation to TGFβ [11]. A similar thing was reported for other analogues [205]. Taken together, this suggests that prostacyclins may be beneficial in the treatment of heritable PAH where BMPRII mutations have been identified. Indeed, only intravenous prostacyclin strongly improved functional class in this group of patients, whereas other therapies were largely in effectual [206].

As already mentioned, the role of PPARβ in vascular remodelling cardiovascular system is not well understood and complex since over expression of PPARβ can drive vascular smooth muscle proliferation and angiogenesis in cancer while PPARβ ligands appear to do the opposite [116,127]. In other studies, PPARβ ligands reduce cardiac hypertrophy and fibrosis in pulmonary artery banding model while having no effect on vascular remodelling in a hypoxia-driven PAH model [207].

Activation of extracellular matrix proteins MMPs are known to contribute to structural remodeling by growth factor activation and degradation of the internal elastic lamina Furthermore, in monocrotaline-treat animals, increases in MMP-9 and MMP-2 protein levels and activity were somewhat suppressed by iloprost treatment, but co-treatment was required with a PDE3/4 inhibitor to have significant effects and return levels to those seen in control animals [38]. The mechanism of suppression is likely to involve cAMP, though PPARα γ and PPARγ ligands are also potent suppressors of MMP-9 and MMP-2 activity [16].

The effects of prostacyclins on remodelling markers in patients is largely unknown though clinical improvement with treprostinil treatment for 12 weeks in a placebo controlled trial was correlated with a reduction in angiopoietin-2 and MMP-9 plasma levels with a trend towards lower PDGF levels [15]. Interestingly, in an experimental model of flow-mediated PH, improvement in right ventricular function with iloprost was associated with a restoration of capillary to myocyte ratio, with no detectible change in vascular remodeling or pulmonary arterial pressure, suggesting new capillary growth may improve a failing heart [208]. It should be noted that this study failed to detect any changes in message levels for VEGF or angiopoietin-2 after iloprost treatment for 28 days. Given that markers were measured at the same time point as histological changes were recorded, may indicate that this was too late to pick up changes associated with new vessel growth.

8. Anti-inflammatory actions of prostacylins

Endothelial dysfunction plays a key role in the development of PAH and this in turn causes the expression of adhesion molecules (e.g. P-selectin, ICAM-1) and the subsequent adherence of platelets and leukocytes to the injured endothelium [14,209,210]. Extensive infiltration of T-lymphocytes, macrophages and dendritic cells occurs in the distal arteries

and plexiform lesions of children and adults with IPAH [13,14,209] culminating in an environment where pro-inflammatory cytokines, particularly interleukins 1 (IL-1) and 6 (IL-6) and chemokines (e.g. CCL2, also known as monocyte chemotactic protein, MCP-1) are up-regulated [7,14]. It is becoming increasingly recognised that the anti-inflammatory actions of PGI_2 may contribute to the beneficial effects of these agents in PAH as well as in critical limb ischaemia, sclerodema and diabetes. PGI_2, iloprost and beraprost are capable of inhibiting the expression of selectins (P and E) and the adhesion molecules ICAM and vascular cell adhesion molecule-1 (VCAM) in endothelial (figure 3) or inflammatory cells of patients with PAH, systemic sclerosis, peripheral vascular disease and type 2 diabetes mellitus [36,172,211,212]. This will all serving to reduce leukocyte–endothelium interactions during inflammation [213]. In diabetic patients treated with beraprost for three years, low VCAM levels were associated with a significantly reduced intimal-media thickness of carotid arteries compared to untreated diabetics [212]. The mechanism associated with adhesion molecule suppression is not well understood, but a number of studies show analogue effects can be mimicked by cyclic AMP and are sensitive to adenylyl cyclase inhibition or Rac1 inhibitors [213-215]. Apart from one study [214], the role of the IP receptor has not specifically been investigated, though given that cicaprost could significantly inhibit TNF-α - and IL-1β-induced cell expression of ICAM-1 and VCAM-1 in cultured human coronary smooth muscle [216] suggests a likely role for this receptor.

Furthermore, analogues can down regulate pro-inflammatory cytokine (TNF-α, IL-1 IL-6 and interferon-γ) and chemokine production (*e.g.* monocyte chemotactic protein 1, MCP-1; granulocyte macrophage colony-stimulating factor, GM-CSF; macrophage inflammatory protein 1, MIP-1) in response to bacterial products (endotoxin) in alveolar macrophages [217], monocytes [218,219], dendritic cells [220] and T-lymphocytes [221]. More often

than not, suppression of proinflammatory cytokines and chemokines in these studies, occurred in a largely, though not exclusively IP receptor driven manner [218,220,221] and in part involving PPARγ [219] though other studies failed to confirm a role for PPARs [218]. NF-κB plays a critical role in the downstream effects of endotoxin, with analogues shown to suppress NF-κB activity in these inflammatory cell types [217,220,221], though one study reported that the mitogen-activator protein kinase pathway (MAPK) may instead be the target [219].

Studies in patients are limited, though iloprost inhibited plasma TNFα levels in critical limb ischemia [222]. In IPAH, epoprostenol treatment reduced elevated circulating levels of MCP-1 [223] and in combination with bostantan, significantly reduced human leukocyte antigen–DR expression, a marker of endothelial cell activation [209]. Whether PPARs play a significant role in analogue suppression of inflammatory mediators remains largely undetermined, though in the lung, PPARα and to a lesser extent PPARγ are key regulators of adhesion molecule expression while both PPARs are major inhibitors of pro-inflammatory cytokine production via transrepression of NF-κB [127,198,204]. By comparison, little is known about the role of PPARβ, but it has been implicated in the antithrombotic effects of treprostinil in human platelets [103].

9. Concluding remarks

PGI_2 can no longer be considered a hormone that just produces its biological effects through activation of the IP receptor. The family of transcription factors knows as PPARs must now be considered a target through which PGI_2 or its stable analogues can modulate cell growth, endothelial cell activation, inflammation and apoptosis and produce beneficial effects in PAH. While PPARs can be activated independently of the IP receptor, few studies have considered the role membrane receptors (or for that matter cAMP) either in

inducing activation or modulating ligand binding. The future challenge will be in identifying the role of specific PPAR isoforms not only the aetiology of PAH, but also in response to analogue activation. The role of VEGF in PAH (vascular protective verses angiogenic) is clearly an area that needs further investigation, in particular whether up-regulation of this growth factor by PGI_2 analogues is detrimental in PAH patients or contributes to lack of efficacy in end-stage disease. Given that the aim of any treatment is to reverse the remodelling process, then the possibility of earlier and aggressive intervention with PGI_2 agonists should be considered. Finally PGI_2 agonists differ in their pharmacological profile, meaning the clinician should not readily assume they are dealing with a homogenous class of agents when it comes to clinical treatment or side-effect profile. It may be that such differences can be exploited in future therapies or be utilised to tease out the role of different prostanoid receptor subtypes. Thus PGI_2 agonists can act upon different receptor targets meaning a similar spectrum of clinical effects cannot be readily be assumed with this class of agents.

Author disclosures

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Table 1. Distinct differences in prostanoid receptor binding affinities (K_i) for prostacyclin mimetics compared with prostaglandin I_2 (PGI_2), prostaglandin E_2 (PGE_2) and prostaglandin D_2 (PGD_2) at human and mouse (rat) prostanoid receptors. Radioligand binding data (K_i in nM) has been taken from original study references for PGI_2 analogues, PGE_2, PGD_2 for selexipag and metabolite [29,75,89,224]. K_i values for PGI_2 against IP & EP_4 receptors come from three individual studies where [^{3}H]-PGI_2 showed saturable binding at a single high affinity site or displaced [^{3}H]-PGE_2 [225-227]. Estimated K_i values EP_1, EP_3, & TP receptors come from historical dose-ratio and binding data where prostacyclin contractile have been directly with PGE_2 [78-80]. Blank means K_i value >3μM, ND means not done, YES indicates evidence for functional activity and NO means the opposite. [a] = K_i value from [89] and GP=guinea-pig.

Table 1. Receptor binding affinities (Ki, nM) of prostacyclin mimetics to human & rodent prostanoid receptors compared with endogenous ligands									
Ligands		**IP**	**DP**	**EP1**	**EP2**	**EP3**	**EP4**	**TP**	**FP**
Cicaprost	Human	17	>1340	>1340	>1340	255	44	>1340	>1340
	Mouse	10		1300		170			
Iloprost	Human	4	1016	1	1172	203 (56)[a]	212		131
	Mouse	11		21	1600	27	2300		
Beraprost	Human	39				680			
	Mouse (rat)	16 (19)				110			
Treprostinil	Human	32	4.4	212	3.6	2505	826		
	Mouse	YES	ND	ND	YES	ND	ND	ND	ND
Selexipag	Human	260							
	Rat	2100							
MRE-269	Human	20	2600						
	Rat	220							
PGI_2	Human	2	ND	≥100	ND	10-40		~100	ND
	Mouse (GP)	17 (16)	ND	~200	ND	12-50	NO	~100	ND
PGE_2	Human		307	9.1	4.9	0.3	0.8		119
	Mouse			20	12	0.8	1.9		100
PGD_2	Human		2		2973	421	1483		7
	Mouse		21			280			47

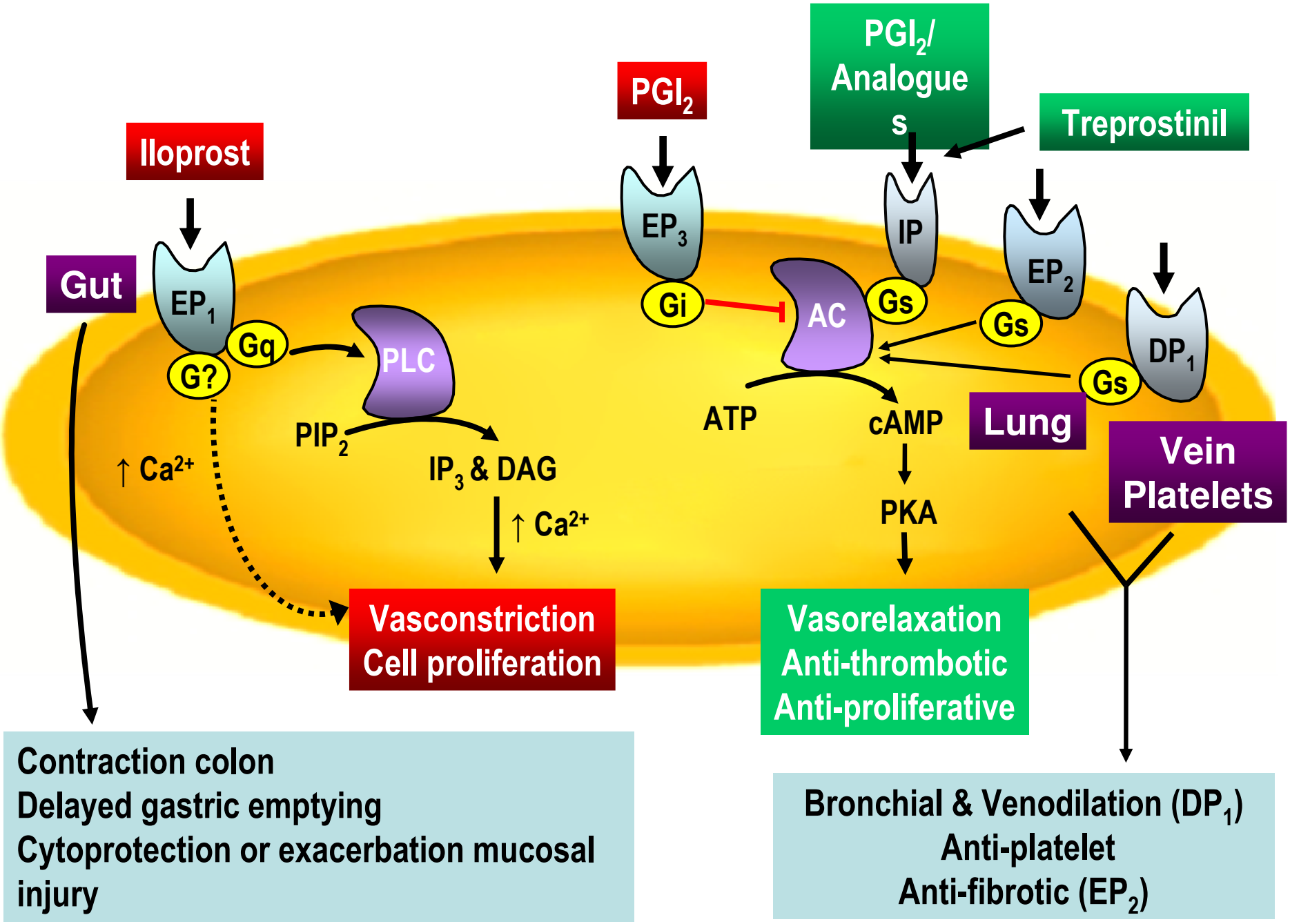
Figure 1. Prostacyclin drugs differentially bind to multiple prostanoid receptors at therapeutic doses. All prostacyclin mimetics, including selexipag and its metabolite, bind to the IP receptor which is coupled via Gs to adenylyl cyclase and cyclic AMP production. Prostacyclin (PGI_2) can also bind to and activate the EP_3 receptors in human pulmonary arteries and platelets and gut (not shown). These receptors counteract IP receptor signalling by lowering cyclic AMP levels through Gi. Iloprost can bind with equipotent affinity to the EP_1 receptor, which elevates intracellular Ca^{2+} though activation of Gq, and an unknown G-protein pathway; the consequence would be to cause vasoconstriction and cell proliferation as well as produce functional effects in the gut as indicated. Treprostinil has 10 fold higher affinity at EP_2 and DP_1 receptors compared to the IP receptor, activation of which will also elevate cAMP. Both these receptors are found in airways, platelets and fibroblasts, and EP_2 receptors along with IP receptors, are expressed in various inflammatory cell types where they reduce inflammation (not shown). AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; G, G protein; IP_3, inositol triphosphate; PKA, protein kinase A; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate.

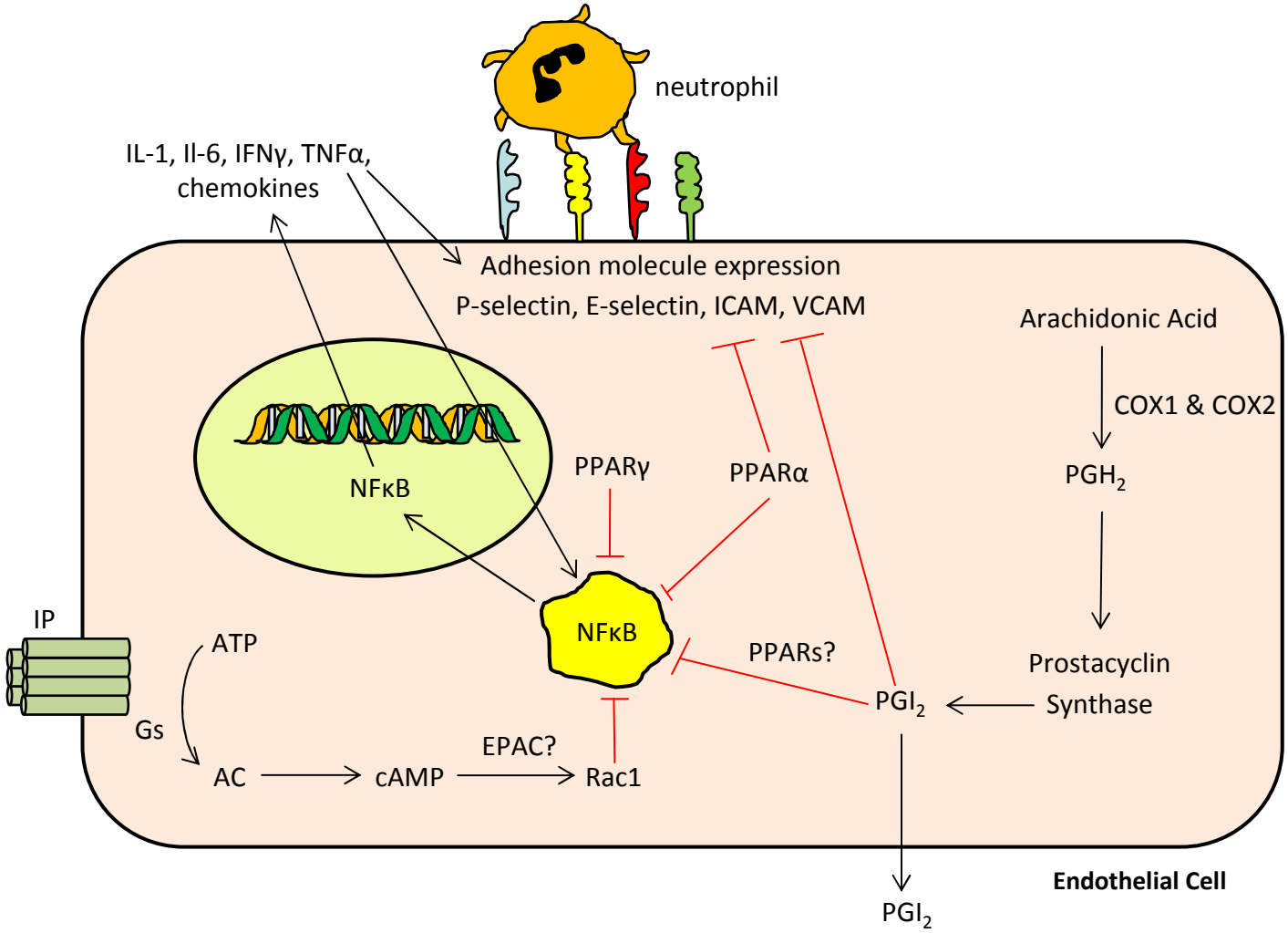
Figure 2. Prostacyclins and PPARγ ligands can enhancing BMPRII signalling in PAH and oppose cell growth through PDGF and TGF-1β receptor pathways. Upon ligand binding, the BMPRII receptor phosphorylates a type I receptor (ALK1, ALK2, ALK3 or ALK6) leading to the phosphorylation of Smad1/5/8. Upon phosphorylation of Smad4, this causes translocation of the phosphorylated Smads to the nucleus to modulate the expression of target genes that inhibit growth and promote apoptosis. Upon TGFβ ligand binding, the TGF-1β type II receptor phosphorylates a type I receptor, ALK5. This leads to phosphorylation of Smad2/3 which phosphorylates Smad4, with the complex translocating to the nucleus. Activation of this receptor will also lead to activation of extracellular signal

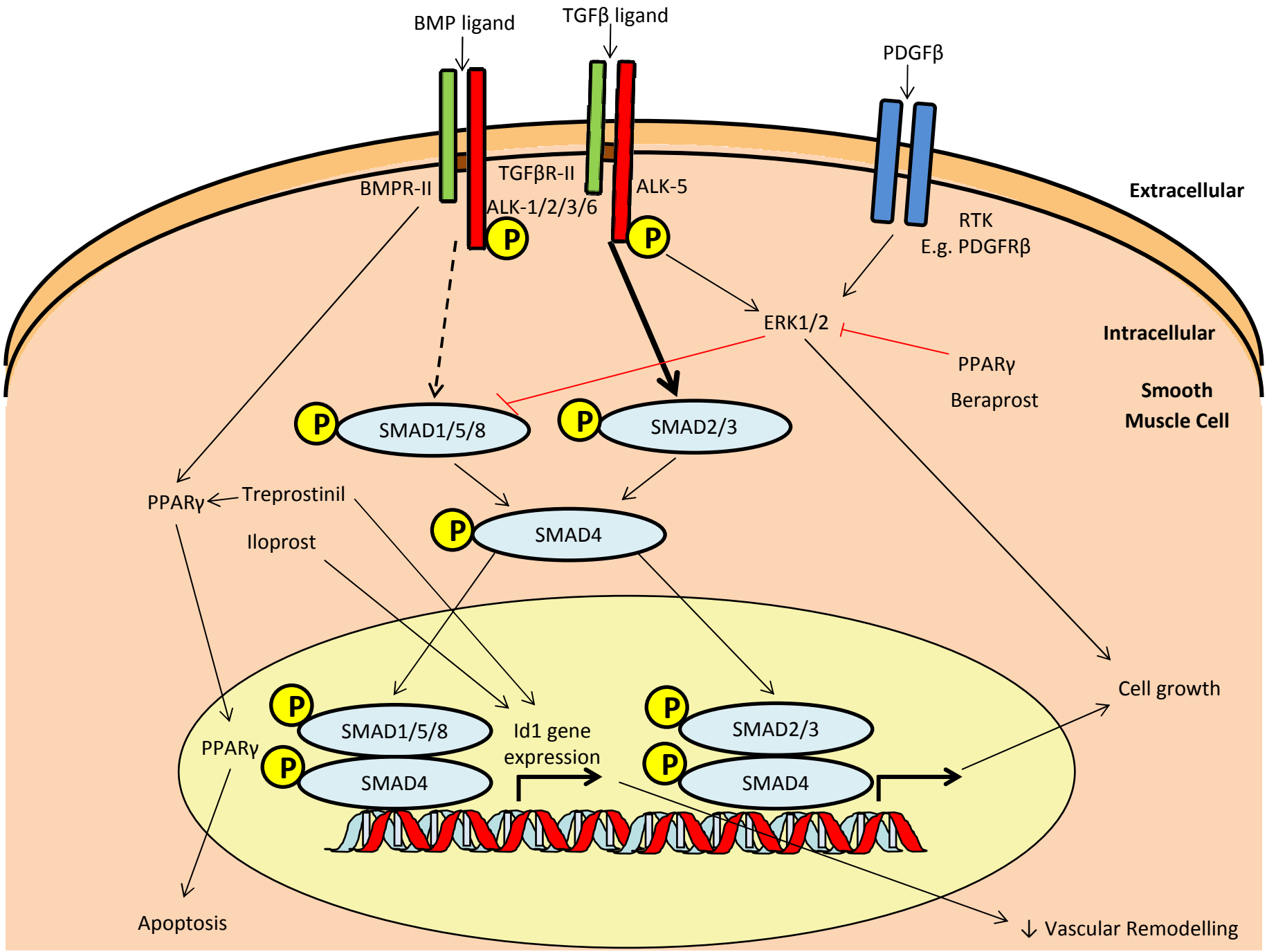
regulated kinases (ERKs), which can be inhibited by PPARγ and prostacyclin analogues. ERK activation limits the phosphorylation of Smad1/5. Stimulation of ERK by receptor tyrosine kinases (RTKs) can also further limit Smad c-terminus phosphorylation. These effects are integrated at the level of inhibitory DNA binding protein 1 (Id1) gene expression, which can also be activated by prostacyclin analogues independently of SMADs.

Figure 3. Anti-inflammatory actions of prostacyclin and PPARs. Endothelial dysfunction associated with the expression of adhesion molecules, adherence of leukocytes to the injured endothelium, and an environment where pro-inflammatory cytokines are produced by endothelial and various inflammatory cell types (not shown), is common place in pulmonary arterial hypertension. TNFα, through activation of the transcription factor, NF-κB is a potent driver of these events and has often been used experimentally to investigate analogue and PPAR driven effects on inflammatory mediators. Prostacyclin and its analogues are potent inhibitors of inflammation, where NF-κB is suppressed through IP receptor activation and may involve Epac-dependent activation of Rac-1 or PPAR activation either through the receptor or via direct binding. The role of PPARs in mediating the effects of prostacyclin is assumed rather than being directly investigated.

AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; Gs, stimulatory G protein; PKA, protein kinase A; COX, cyclooxygenase; EPAC, exchange protein activated by cAMP; ICAM, intercellular adhesion molecule; IFγ, interferon-γ; IL-1, interleukin-1; IL-6, interleukin-6; IP, prostacyclin receptor; NF-κB, nuclear factor kappa B; PGI_2, prostacyclin; PGH_2, prostaglandin H_2; PKA, protein kinase A; PPAR, peroxisome proliferator-activated receptor α, γ; Rac-1, Ras-related C3 botulinum toxin substrate 1; TNF-α, tumour necrosis factor; VCAM, vascular cell adhesion molecule-1.


Iloprost
Gut
EP1
Gq
G?
PLC
PIP2
IP3 & DAG
↑ Ca2+
↑ Ca2+
Vasconstriction
Cell proliferation
Contraction colon
Delayed gastric emptying
Cytoprotection or exacerbation mucosal injury
PGI2
EP3
Gi
AC
ATP
cAMP
PKA
PGI2/ Analogues
Treprostinil
IP
EP2
DP1
Gs
Gs
Gs
Lung
Vein
Platelets
Vasorelaxation
Anti-thrombotic
Anti-proliferative
Bronchial & Venodilation (DP1)
Anti-platelet
Anti-fibrotic (EP2)


neutrophil
IL-1, Il-6, IFNγ, TNFα,
chemokines
Adhesion molecule expression
P-selectin, E-selectin, ICAM, VCAM
Arachidonic Acid
COX1 & COX2
PGH2
NFκB
PPARγ
PPARα
NFκB
IP
ATP
Gs
PPARs?
Prostacyclin
Synthase
PGI2
AC
cAMP
EPAC?
Rac1
Endothelial Cell
PGI2


BMP ligand
TGFβ ligand
PDGFβ
BMPR-II
TGFβR-II
ALK-1/2/3/6
ALK-5
Extracellular
RTK
E.g. PDGFRβ
ERK1/2
Intracellular
PPARγ
Beraprost
Smooth
Muscle Cell
SMAD1/5/8
SMAD2/3
PPARγ
Treprostinil
Iloprost
SMAD4
Cell growth
SMAD1/5/8
SMAD2/3
Id1 gene
expression
PPARγ
SMAD4
SMAD4
Apoptosis
↓ Vascular Remodelling