Role of Prostacyclin versus Peroxisome Proliferator-Activated Receptor β Receptors in Prostacyclin Sensing by Lung Fibroblasts

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Prostacyclin and its mimetics are used therapeutically for the treatment of pulmonary hypertension. These drugs act via cell surface prostacyclin receptors (IP receptors); however, some of them can also activate the nuclear receptor peroxisome proliferator-activated receptor β (PPARβ). We examined the possibility that PPARβ is a therapeutic target for the treatment of pulmonary hypertension. Using the newly approved (for pulmonary hypertension) prostacyclin mimetic treprostinil sodium, reporter gene assays for PPARβ activation and measurement of lung fibroblast proliferation were analyzed. Treprostinil sodium was found to activate PPARβ in reporter gene assays and to inhibit proliferation of human lung fibroblasts at concentrations consistent with an effect on PPARs but not on IP receptors. The effects of treprostinil sodium on human lung cell proliferation are mimicked by those of the highly selective PPARβ ligand GW0742. There are no receptor antagonists for PPARβ or for IP receptors, but by using lung fibroblasts cultured from mice lacking PPARβ (PPARβ$^{-/-}$) or IP (IP$^{-/-}$), we demonstrate that the antiproliferative effects of treprostinil sodium are mediated by PPARβ and not IP in lung fibroblasts. These observations suggest that some of the local, longer-term benefits of treprostinil sodium on reducing the remodeling associated with pulmonary hypertension may be mediated by PPARβ. This study is the first to identify PPARβ as a potential therapeutic target for the treatment of pulmonary hypertension, which is important because orally active PPARβ ligands have been developed for the treatment of dyslipidemia.

Keywords: fibroblast; nuclear receptors; PPARβ; prostacyclin; pulmonary hypertension

Pulmonary hypertension is defined clinically as a mean pulmonary artery pressure value exceeding 25 mm Hg at rest or 30 mm Hg during exercise. Pulmonary hypertension is caused when there is an imbalance in the release of vasoactive mediators that favor vasoconstriction, which stimulates the release of growth factors and subsequent vessel wall (remodeling). Remodeling in pulmonary hypertension is characterized by hypertrophy and hyperplasia of the various cells types within the vessel, including fibroblasts, medial smooth muscle cells, and endothelial cells (1). In this respect, pulmonary fibroblasts are important because their migration and differentiation into the vessels contributes significantly to the formation of the neointima. Arteries within the lung become narrow, creating increased resistance and blood pressure and thereby increasing workload for the heart. The prognosis for patients with pulmonary hypertension is usually poor, with survival rates of 68% at 1 yr, 48% at 3 yr, and 34% at 5 yr (2).

Pulmonary hypertension is treated with a number of therapeutic interventions (1), including administration of the vasodilator prostacyclin. Prostacyclin has a short half-life (2–3 min) and undergoes spontaneous hydrolysis into 6-keto $PGF_{1\alpha}$ (3); because of this, therapeutic administrations need to be via continuous intravenous infusion. Most recently, the stable prostacyclin mimetic, treprostinil sodium (Remodulin; United Therapeutics Corp., Silver Spring, MD), has been developed for the treatment of pulmonary hypertension. Treprostinil sodium is stable at room temperature and has a neutral pH, which allows subcutaneous infusion.

Prostacyclin and its analogs activated G protein–coupled cell-surface prostacyclin (IP) receptors. Activation of IP receptors stimulates adenylyl cyclase, leading to an increase in cyclic adenosine monophosphate, which inhibits cell proliferation and inflammatory mediator release (4). However, prostacyclin and some mimetics are capable of activating the peroxisome proliferator-activated receptor β (PPARβ) (3). PPARs are a family of three nuclear receptors (PPARα, PPARβ/δ, and PPARγ) that have influences on many biological processes, including lipid and energy metabolism (5, 6), epidermal wound repair (7), inflammation responses, and atherosclerotic plaque formation. The literature describes the function of PPARγ or PPARα in physiologic and pathophysiologic responses, but much less is known about the role of PPARβ in health and disease. Nevertheless, activation of PPARβ has been found to have anti-inflammatory properties via interactions with the transcriptional repressor BCL-6 (8).

The potential role of IP versus PPARβ as therapeutic targets in pulmonary hypertension has not previously been addressed. There are no antagonists of IP or PPARβ receptors. In the current study, we used selective ligands and cells grown from the lungs of mice deficient in IP (9) or PPARβ (10) to address this question. We show that treprostinil sodium is a PPARβ ligand and that its antiproliferative effects on lung fibroblasts are mediated by PPARβ and not by IP receptors.

MATERIALS AND METHODS

Origin and Genotyping of Genetically Modified Mice

IP receptor and PPARβ/δ gene-deleted (IP$^{-/-}$ and PPARβ/δ$^{-/-}$) mice were generated as previously reported (9, 10). Both types of genetically modified mice were back bred for seven generations with C57 BLK 6 mice. To prepare cultures of lung cells, animals were killed, and their lungs were removed. The lungs were placed in sealed sterile culture

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tubes containing sterile Dulbecco's modified Eagle's medium (DMEM) with penicillin/streptomycin (100 U/ml), L-glutamine (2 mM), nonessential amino acids (1%), and amphotericin B (2.5 μg/ml) and shipped by overnight courier. Lungs taken from C57 BLK 6 mice and treated in the same way were used as controls. The tissue was genotyped by PCR. Details on the methods can be found on the online data supplement.

Murine Cell Culture

Lung fibroblasts are an important cell target for antiproliferative agents in the treatment of pulmonary hypertension. We used primary cultures of human or murine lung fibroblasts to address our questions. Lungs were washed with sterile PBS, chopped, and placed in culture flasks in supplemented DMEM. Tissue was incubated in culture until cells had explanted and achieved more than 80% confluence. Cells were seeded onto separate 96-well plates at 40% (20,000 cells/ml) confluence. After 24 h, the medium was replaced with fresh DMEM without FCS but with BSA (0.1%). Drugs were added 2 h later. After 4 h, proliferation was initiated by the addition of FCS (3%). Cells were incubated in an atmosphere of 5% CO_2 in air at 37°C for 72 h, after which time proliferation was quantified using the CyQUANT assay (11).

Primary Cultures of Human Lung Fibroblasts

Human lung fibroblasts (American Type Culture Collection CCL-211) were grown in supplemented DMEM containing 10% FCS. Cells were plated at 20,000 cells/ml into 96-well plates. At 40% confluence, the cells were growth arrested (no FCS) and left for 24 h. Drugs were added and incubated for 4 h, and cells were stimulated with the addition of FCS (3%). Cells were incubated in at 37°C for 5 d, and proliferation was quantified using the CyQUANT assay.

Measurement of Cell Number

A CyQUANT cell proliferation assay was used to determine cell proliferation. All medium was removed from the cells, which were frozen and stored at −80°C for 1 h. Cells were thawed, lysed, and treated with a fluorescent dye according to manufacturer's instructions. Visual scoring was performed on all experiments before fluorescence assay. Data were observed as a percentage of confluence. MTT assay was also used to confirm cell viability after incubation with ligands. Details can be found in the online data supplement.

Western Blotting

Standard protocols were used to blot for PPARβ in cells. Details can be found in the online supplement.

PPAR Reporter Gene Assay

HEK293 cells were seeded onto six-well plates in 10% DMEM. At 50% confluence, cells were transfected with the desired plasmids (Dre-1 [p4xDRE-luc] and pcDNA or Dre-1 and PPARβ [pCMX-mPPARb/d] or PPARγ [pCMX-mPPARg]; 2 μg total). Plasmids were initially made up to 200 μl of serum-free DMEM. Transfection reagent (NovaFECTOR) (4 μl) was added, and the mixture was incubated at room temperature for 15 min to allow for binding of plasmid to transfection reagent. Just before transfection, cells were washed with PBS, and 800 μl of DMEM was added. The plasmid–reagent complex was added to each corresponding well dropwise and incubated for 3 h at 37°C. Medium was removed, and cells were washed with PBS, trypsinized, and transferred onto 96-well plates at 100% confluence in DMEM containing 10% serum. Cells were incubated overnight before the addition of drugs. Drugs were incubated overnight. The supernatant was removed, and 50 μl of reporter lysis buffer (Promega, Southampton, UK) was added to each well and incubated at room temperature for 15 min. Twenty-five microliters of this sample was used to measure protein (BCA), and 25 μl was used to measure reporter gene activation in a luciferase assay as previously described (12, 13).

Reagents

DMEM, BSA, and Mayer's hematoxylin stain were purchased from Sigma (Dorset, UK); all other cell culture materials and pcDNA were purchased from Invitrogen (Paisley, UK). CyQUANT cell proliferation assay kit was from Cambridge BioScience Ltd. (Cambridge, UK), and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15 d-PGJ_2) was purchased from Biomol (Plymouth Meeting, PA). GW0742 and treprostinil sodium were kind gifts from Timothy M. Willson (GlaxoSmithKline, Research Triangle Park, NC) and Carl Sterritt (United Therapeutics Corp.), respectively. Rosiglitazone was purchased from Alexis (Nottingham, UK). Rabbit anti-PPARβ/δ antibody was from Abcam (Cambridge, UK), and goat anti-rabbit antibody was from Dako (Glostrup, Denmark). ECL detection reagent and hyperfilm were from Amersham Biosciences (Buckinghamshire, UK). NovaFECTOR was from VennNova (Pompano Beach, FL). P4xDRE-luc reporter gene was a gift from Bert Vogulstein at Johns Hopkins University (Baltimore, MD), pCMX-mPPARβ plasmid was a gift from Ron Evans at the Salk Institute (San Diego, CA), and pCMX-mPPARγ was a gift from Christopher Glass at the University of California (San Diego, CA).

Statistics

All results are represented in the text and figures as mean ± SEM. Statistical differences between groups of data were tested using appropriate tests, which are described in the relevant figure legends. A P value of < 0.05 was considered to be statistically significant.

RESULTS

Effects of Treprostinil Sodium, Traditional IP Ligands, and Traditional PPAR Ligands on Human Lung Fibroblast Proliferation

Treprostinil sodium inhibited, in a concentration-dependent manner, the proliferation of human lung fibroblasts induced by a submaximal concentration of FCS (3%). Inhibitory effects became statistically significant at concentrations of 10^{-7} M and higher (Figure 1). Similarly, the PPARβ-specific ligand GW0742 inhibited human lung cell proliferation, with two clear concentration-related effects being noted. At low concentrations, GW0742

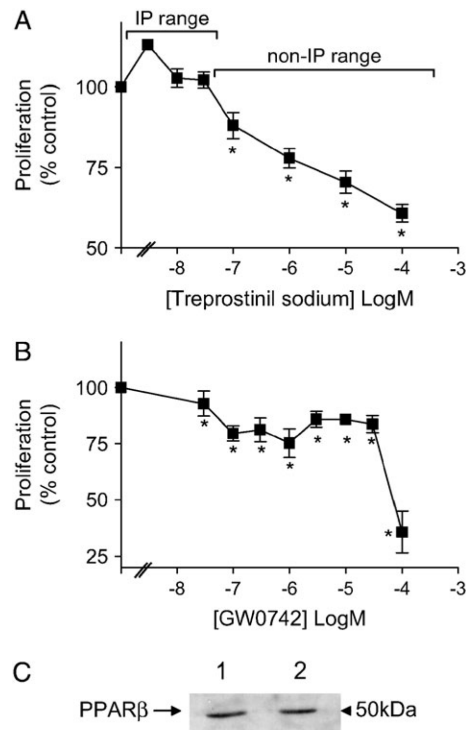

Figure 1. Importance of IP versus PPARβ in human lung fibroblast proliferation. (*A*) Effect of treprostinil sodium and (*B*) GW0742 on human lung fibroblast proliferation. Proliferation is expressed as percentage of control response induced by 3% FCS. Data are means ± SEM; $n = 9$. *$P < 0.05$ by one sample t test. (*C*) Expression of PPARβ protein in human lung fibroblasts using cells cultured from two separate donors.

inhibited proliferation in a concentration-dependent manner, which was maximal at 10^{-6} M (25 ± 6% inhibition) (Figure 1). At high concentrations (10^{-4} M), GW0742 induced an additional and dramatic inhibition of cell proliferation. All results were confirmed by visual scoring of cells and by MTT assay, which showed a reduction in cell number and no effects of cell death (data not shown). Western blotting demonstrated that human lung fibroblasts contain PPARβ (Figure 1). In addition to treprostinil sodium (Figure 1), cicaprost, iloprost, and carbaprostacyclin inhibited proliferation of human lung fibroblasts but at concentrations above those expected to activate IP receptors (10^{-4} M) (Figure 2). In addition to the PPARβ ligand GW0742, the putative endogenous PPAR ligand 15 d-PGJ_2 and the PPARγ ligand rosiglitazone inhibited human fibroblast cell proliferation (Figure 2). However, the PPARα ligand WY-14643 or the mixed PPARα/PPARβ ligand bezafibrate had no inhibitory effects on cell proliferation (Figure 2).

Role of PPARβ versus IP Receptors in the Normal Growth and Morphology of Murine Lung Fibroblasts

The genotypes of $IP^{-/-}$ and $PPAR\beta^{-/-}$ mice were confirmed by PCR (Figure 3). Lung cells cultured from each type of mouse seemed to be similar in morphology to those from control wild-type (C57 BLK 6) mice, with a typical fibroblast phenotype (Figure 3). Lung fibroblasts cultured from control mice and from $PPAR\beta^{-/-}$ mice proliferated in response to FCS similarly, whereas lung fibroblasts from $IP^{-/-}$ mice seemed to proliferate at approximately twice the rate of the control cells. As seen in human lung fibroblasts, the prostacyclin mimetics treprostinil sodium (Figure 4), cicaprost, or carbaprostacyclin (10^{-4} M; data not shown) inhibited proliferation of murine lung fibroblasts. The inhibitory effects of treprostinil sodium on proliferation were significantly reduced in cells derived from $PPAR\beta^{-/-}$ animals (Figure 4). No significant differences were seen in the ability of treprostinil sodium to inhibit proliferation in cells from control versus $IP^{-/-}$ mice (Figure 4). The inhibitory effects of rosiglitazone on lung fibroblast proliferation were unaffected by PPARβ (Figure 4B) or IP gene deletion (data not shown). Treprostinil sodium had no effect on cell viability at concentrations up to 10^{-4} M measured by MTT (data not shown).

Effects of Treprostinil Sodium of PPARβ/δ versus PPARγ Receptor Activity

Plasmids for PPARβ or PPARγ could not be successfully transfected into primary cultures of pulmonary fibroblasts (human or murine). We therefore performed reporter gene assays in HEK293 cells. Treprostinil sodium (Figure 4) or GW0742 (data not shown) but not rosiglitazone (data not shown) increased PPARβ in a reporter gene assay. By contrast, treprostinil sodium had no effect on PPARγ activation (Figure 4).

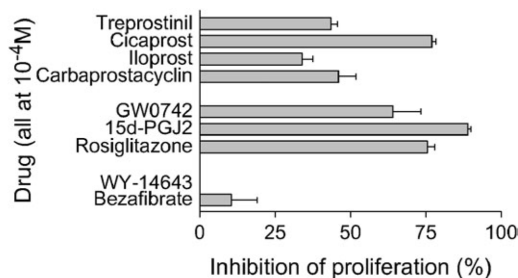

Figure 2. Effects of prostacyclin or PPAR ligands on proliferation of human lung fibroblasts. Each drug was used at 10^{-4} M. Proliferation is inhibition of control response induced by 3% FCS. Data are means ± SEM. $n = 9$ for treprostinil sodium, GW0742, and 15 d-PGJ_2; $n = 6$ and for rosiglitazone and bezafibrate; and $n = 3$ determinations for all other mimetics.

DISCUSSION

The treatment of pulmonary hypertension is aimed at two pathologies: (*1*) the vasoconstriction and (*2*) the profound remodeling that characteristically mediates lung vessel narrowing. Prostacyclin therapy can reduce vasoconstriction in pulmonary hypertension, but its key mode of action is on reducing vascular remodeling. Lung fibroblasts, which infiltrate and proliferate within the blood vessel (14–16), are important target cells for antiproliferative treatments used for pulmonary hypertension. Prostacyclin is thought to relieve some of the symptoms of pulmonary hypertension via an action on cell-surface IP receptors. However, some prostacyclin mimetics are also ligands for the nuclear receptor PPARβ. In the current study, we demonstrate, for the first time, that PPARβ is expressed in human lung fibroblasts and that its activation leads to reduced proliferation. Using cells from genetically modified animals, we show that some of the antiproliferative effects of treprostinil sodium, which was recently approved for the treatment of pulmonary hypertension, are via PPARβ.

We found that the proliferation of human lung fibroblasts was inhibited by a number of prostacyclin mimetics, including the newly introduced treprostinil sodium. In contrast to observations using vascular smooth muscle cells (17), the inhibitory effects of treprostinil sodium (and other mimetics) were seen only at concentrations above those associated with activation of IP receptors (i.e., $> 10^{-7}$ M) but within the range observed for prostacyclin mimetics on PPARβ receptors (i.e., $> 10^{-6}$ M) (18). These data suggest that although IP receptors may mediate the vasodilator properties of prostacyclin and its mimetics, it is not the receptor target that mediates inhibition of fibroblasts growth. We found that human lung fibroblasts expressed detectable levels of PPARβ and that proliferation of these cells was inhibited by the highly selective PPARβ ligand GW0742. Proliferation of these cells was also inhibited by PPARγ, but not PPARα, ligands. These observations suggest that treprostinil sodium, like some other prostacyclin mimetics, could be a ligand for PPARβ. This was confirmed in the current study using our standard reporter gene assay (12). By contrast, using similar reporter gene assays, we show that treprostinil sodium did not activate PPARγ. Because PPARα ligands did not limit proliferation in these cells, it seems clear that, in the micromolar range, treprostinil sodium inhibits lung fibroblast proliferation by an action on PPARβ.

There are no selective antagonists of PPARβ or IP receptors. Therefore, we used lung fibroblasts cultured from mice deficient in either receptor to further identify the role of each in proliferation. As seen with human cells, proliferative responses in murine lung fibroblasts cultured from control animals were inhibited by treprostinil sodium and other IP receptor ligands. Similarly, the inhibitory effects were seen only at high concentrations, suggesting that IP receptors were not involved in the effects of treprostinil sodium on lung cell proliferation. This hypothesis was substantiated by our findings that the inhibitory effects of treprostinil sodium were unaffected by IP receptor deletion. However, the inhibitory effects of treprostinil sodium were reduced in cells derived from $PPAR\beta^{-/-}$ animals. These observations strongly suggest that the prostacyclin mimetics can inhibit lung fibroblast proliferation independently of IP receptors and via activation of PPARβ, in accordance with previous observations using other cell types (17, 19). Deletion of the IP receptor resulted in an increased amount of proliferation in the lung

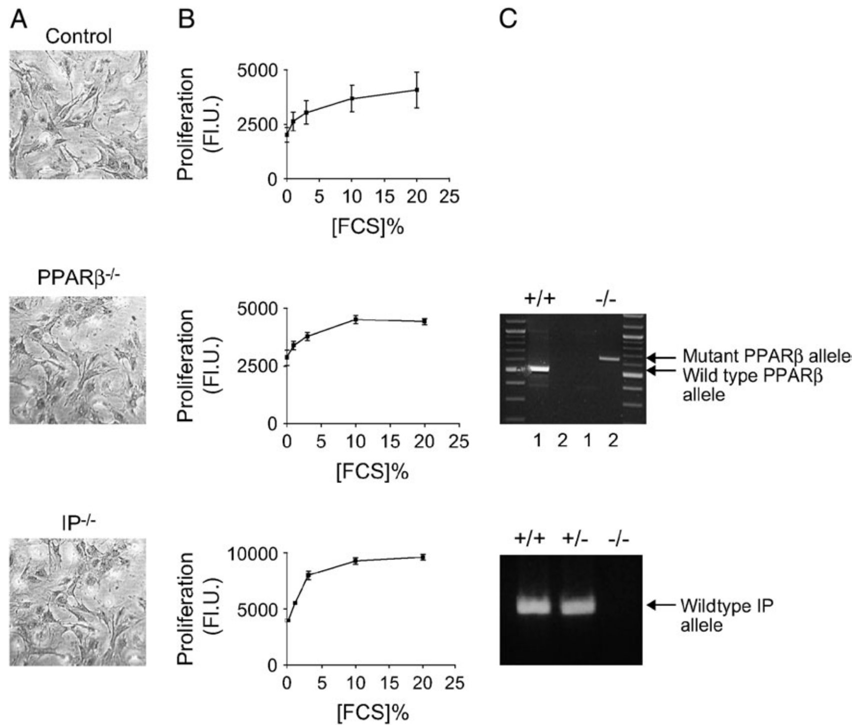

Figure 3. Phenotype and genotype characterization of murine lung fibroblasts cultured from control wild-type mice or from mice lacking IP or PPARβ genes. (*A*) Morphology of murine lung fibroblasts. Cells were grown in a sterile 96-well plate, washed with PBS (without calcium and magnesium), and incubated at room temperature for 10 min with Mayer's hematoxylin stain. *Original magnification*: ×10. (*B*) Proliferative responses of cells from each type of animal in response to FCS (0–20%). The amount of fluorescence detected is directly proportional to the amount of DNA in each sample. Data are means ± SEM; $n = 6$ experiments using cells grown from two animals for each cell type. (*C*) PCR genotyping of genetically modified mice. For genotyping of PPARβ, PCR reactions were performed using primers allowing an amplification of a 500-bp DNA fragment of the wild-type PPARβ/δ allele and no amplification of the mutant allele or primers allowing the amplification of a 650-bp DNA fragment of the mutant allele and no amplification of the wild-type allele. For genotyping of $IP^{-/-}$ and control wild-type mice, PCR reactions were performed from genomic DNA isolation from mice toes. PCR reactions were performed using primers for the wild-type allele, which result in a 425-bp product.

fibroblasts compared with wild-type cells, indicating that the IP receptor may serve a role in controlling the proliferation of lung fibroblasts. However, the increased rate of $IP^{-/-}$ cells was not deterred in the presence of indomethacin, which blocks prostacyclin production via an action on cyclo-oxygenase-1 or cyclo-oxygenase-2 (20). This suggests that the increased proliferation is not dependent on an interaction of the receptor with prostacyclin *per se*, but rather is due to some intrinsic activity or to an unrelated phenotypic difference in these cells.

Prostacyclin and related therapy is the gold standard for the treatment of pulmonary hypertension. Many of the benefits of prostacyclin in this disease are associated with the arrest and reversal of the profound remodeling that occurs in the pulmonary vascular wall. The observations presented in this article suggest that prostacyclin mimetics limit lung fibroblast proliferation via an action of PPARβ receptors. However, the therapeutic concentrations of these drugs in the treatment of pulmonary hypertension are in the nanomolar (IP receptor) range. The effects of prostacyclin mimetics on blood pressure (via IP receptor pathways) limits the amount of drug that can be delivered safely. Prostacyclin therapy has another important limitation: It must be give by injection or infusion. The data presented in this article suggest that some of the effects of prostacyclin-like therapies may occur independently of the classical IP receptor and at the level of PPARβ activation. These observations suggest that PPARβ may be a novel therapeutic target for the treatment of pulmonary hypertension, which is important because orally active agonists of PPARβ have already been synthesized and are in development for the treatment of dyslipidemia.

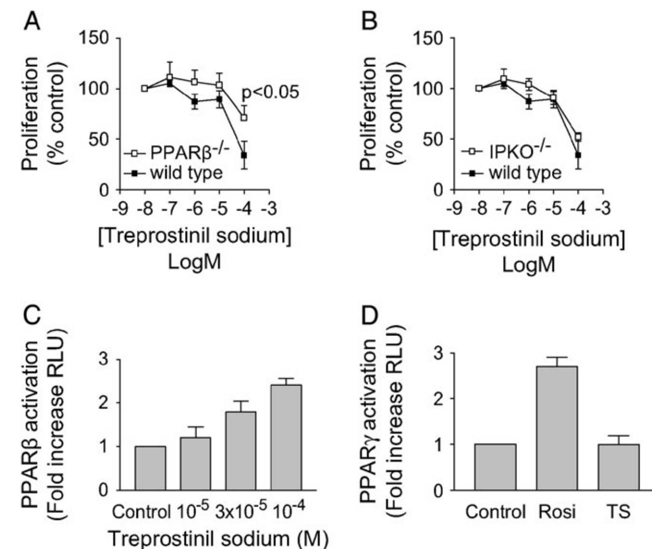

Figure 4. Role of PPARβ versus IP in the effects of treprostinil sodium on lung cell proliferation. Proliferation responses in cells from control or $PPAR\beta^{-/-}$ mice (*A*) or $IP^{-/-}$ mice (*B*) were induced by 3% FCS and are expressed as percentage of control. Data are means ± SEM; $n = 6$ for each data point. $^{*}P < 0.05$ by two-way ANOVA. Effects of treprostinil sodium (TS) on PPARβ (*C*) or PPARγ (*D*) Luciferase reporter gene activity in HEK293 cells. Data show fold activation normalized to control relative light units. Data are means ± SEM; $n = 3$–8.

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