Smooth Muscle Proliferation and Role of the Prostacyclin (IP) Receptor in Idiopathic Pulmonary Arterial Hypertension

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Online Data Supplement

Methods, results and figure legends for online supplement

Patient Characteristics

Lung tissue was taken after subjects or relatives gave informed consent and had Ethics Committee approval from Great Ormond Street (ICH and GOSH REC 05/Q0508/45), Papworth Hospital (REC H00/531/T) and Brompton & Harefield Trust (NHLI REC 01-210) through Dr Wharton (Imperial College, London). Samples were obtained from patients with IPAH undergoing transplant after failed treatment (6 children and 4 adults) or who had been on no therapy (8 children). Treated children (mean age 5.3 years) received i.v. epoprostenol (38-50 ng/kg/min) for at least 1.3 years (in primary cells lines this averaged 3 years) combined with bosentan for more than 1 year. Adult patients (mean age 39.5 years) were on varying prostacyclin therapy with one patient receiving IV epoprostenol (7 months), one nebulized iloprost (1.3 years) and one IV iloprost followed by subcutaneous remodulin (1.6 years), combined with bosentan in two patients. One IPAH adult was known to carry a mutation (N903S) in the bone morphogenetic protein receptor type II (BMPRII) (1). For the treated patients, average mean pulmonary arterial pressure (PAP) was 72±4 mHg (n=6) in children and 55±2 mHg (n=3) in adults. For controls, tissues was obtained from normal children (n=5) and from adults undergoing transplant or lung resection for suspected malignancy (n=6).

Culture of PASMCs from hypertensive and normal patients

PASMCs were isolated from the explanted lung of three children suffering from IPAH who received a bilateral lung or heart/lung transplant. The explanted lungs were bathed in ice cold phosphate buffered saline (PBS) and peripheral pulmonary arteries (o.d. ~250 μm) removed and placed in fresh PBS containing penicillin (300 μg/ml), streptomycin (300 μg/ml) and gentamycin (180 μg/ml). Cells were dissociated by incubation at 37˚C in DMEM/F12 (Invitrogen, Paisley UK) medium buffered with HEPES (pH 7.4) that contained a protease cocktail of 0.125 mg/ml

elastase (Lorne Laboratories, Reading, UK), 0.25mg/ml collagenase (Sigma-Aldrich, Poole, Dorset, UK), 0.06 mg/ml trypsin inhibitor (Sigma) and 3.75 mg/ml bovine serum albumin (BSA). After continuous agitation for 30-45 min, dissociated cells were sedimented by centrifugation at 350g and re-suspended in SmGM complete medium (Lonza Biologics, Slough, GB) containing 10% FBS. Cells were plated into 25 cm^2 flasks and after reaching confluence, were washed with PBS and treated with trypsin-EDTA (Invitrogen) for further passage in DMEM/F12 with 10% FBS. Immunohistochemistry and Western blotting were used to identify the cellular phenotype and confirm the presence of the smooth muscle specific markers α-smooth muscle actin (α-SM actin, clone 1A4, Sigma), smooth muscle myosin heavy chain (MHC, clone hSM-v, Sigma) and caldesmon (clone hHCD, Sigma). Primary human PASMCs were isolated from peripheral blood vessels as previously described (2) from four adults with IPAH and 6 control adults. For comparison, control intrapulmonary PASMCs derived from a 2.5 month old child were obtained from Lonza Group Ltd (Basel, Switzerland). Only cells between passages 3-10 were used.

PASMCs generated wild-type and $IP^{-/-}$ mice

Distal PASMC from homozygous IP receptor-deficient (n=6) and wild type (n=7) mice were isolated, cultured and characterised (by immunostaining). Following midline thoracic incision and exposure of thoracic organs, the right venticle and left atrium were incised. The pulmonary vasculature was perfused with 10ml of warm phosphate-buffered saline (PBS) via right ventricular cannula to remove residual red blood cells. This was followed by infusion of a suspension of iron oxide particles (0.5% wt.vol) and low-melting-point agarose (0.5% wt.vol) in culture medium (10mls at 45^0C). Culture medium consisted of Dulbecco's modified eagles medium supplemented with penicillin (100U/ml), streptomycin (100μg/ml) and amphotericin B (2μg/ml). The trachea was cannulated and lungs inflated with warm (45^0C) culture medium containing 1% (wt/vol) agarose.

The trachea, both lungs and the heart were then dissected free, removed *en bloc* and kept on ice for 10 minutes in culture medium with 10% fetal calf serum (FCS) to set the agarose.

Individual lung lobes were separated from remaining tissue. Subpleural lung sections were taken to a depth of no greater than 1mm using a sterile surgical scalpel blade. Sections were minced with sterile scissors and tissue digested in type II collagenase (80U/ml) for 30 minutes at 37^0C. Subpleural tissue was then homogenised by serial passage through sterile needles of reducing gauge. Cells were transferred to 12 x 75-mm glass tubes (Pyrex, Bibby Sterilin Ltd.) and peripheral PASMC separated using a magnetic separator (Promega, Madison, WI). Cells were repeatedly washed with warm (37^0C) culture medium containing 20% FCS until a clear discard solution was obtained. Magnetically-separated cells were resuspended in 1ml of culture medium (with 20% FCS) and then transferred to 25 cm^2 sterile culture flasks for incubation and culture.

HEK-293 cell lines and culture

A HEK-293 cell line stably expressing the human IP receptor gene (HEK-293-IP) or the empty vector (HEK-293-Zeo) was generated as previously described (3). Two individual zeocin resistant colonies per cell type were isolated and maintained in minimal essential medium (MEM) containing Earle's salts and L-glutamine (Invitrogen, Paisley, UK) supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin (from a 10,000 units/ml stock) (Invitrogen) and Zeocin (400 μg/ml) (Invitrogen).

Cell proliferation assays

For cell counting, HEK-293 and HPASM cells were seeded onto 6-well plates at a density of 0.5-2 $\times 10^4$ cells/ml and grown for 24 hr in the appropriate media. Cells were subsequently starved for 48 hr in a low serum medium containing either 0.1% FBS or, in the case of HEK-293-Zeo cells, no

added serum. To assess the growth characteristics of the different cell lines, cells were incubated in MEM (HEK-293 cells) or DMEM/F12 (PASMCs) containing 10% FBS in the absence and presence of the relevant test agent. Cells were counted at 24 hr intervals (up to 6 days) using an automated cell counter (Sysmex F-520P; Malvern Instruments Ltd, UK) and results expressed as the fold increase in cell number relative to that measured at the end of the starving period (t=0). To assess growth at a single time point, cells were incubated for 48 or 96 hr in media containing either 10% FBS (± treatment or solvent) and or low serum (control). Data were expressed as % change in cell proliferation relative to the growth response induced by 10% FBS.

Determination of [^{3}H]thymidine incorporation into distal PASMC from wild-type and IP receptor–deficient mice was performed as previously described (4). Briefly PASMC were grown to confluence in 75 cm^2 flasks and then sub-cultured in 48-well plates at a seeding density of 5,000 cells/ well in 0.5 ml DMEM containing 10% FBS. Upon reaching a semi-confluent state, the medium was removed, cells washed twice in PBS before being replaced by DMEM containing 0.1% FBS for 48 hr. Treatment groups (4 wells/group) included:

(a) Vehicle (0.5ml/well DMEM with 0.1% FCS+ 0.25μCi/ well methyl ^{3}H-thymidine)

(b) Vehicle + 10ng/ml PDGF-BB

(c) Vehicle + 10ng/ml PDGF-BB + treatment of interest

After 24 hr, plates were washed three times in cold (4^{0}C) PBS, removed then cold 10% trichloracetic acid (TCA) added and samples kept at -4^{0}C for 45 minutes (to remove nucleoproteins). TCA was then replaced with warm sodium hydroxide (0.2M) to lyse cells. After overnight storage at 4^{0}C, 350μl samples were taken from each well and transferred to vials containing scintillation fluid (Ultima Gold^{TM}). Beta emissions were recorded (2 minutes/sample) using Trigard X scintillation counter (Packard Bioscience).

RT-PCR

Messenger RNA was isolated using an RNeasy Mini kit (Qiagen, Crawley, Surrey, UK) and following the manufacturer instructions. DNA polymerase SuperScript™II reverse transcriptase (Invitrogen) was used to synthesise 20 µl of complementary DNA from 5 µg of total RNA as per manufacturer's instructions. For PCR, 2 µl of cDNA was used in a 20 µl reaction and the following added: 5 µl PCR buffer (x10, containing 4 mM $MgCl_2$) (Invitrogen), 1µl deoxynucleoside-5'-triphosphate solution (containing 25 mM of each dNTP), 1 unit Taq DNA polymerase (Invitrogen) and 1 µM of each specific primers. Primers for the human IP receptor (accession number NM_000960) were designed to span across two exons and were: 5' ATGTACCGCCAGCAGAAGCG 3' (nt 750-769) in the forward direction and 5' GAAGCGGAAGGCAAGGAGCTC 3' (nt 934-953) in the reverse direction. Primers for human GAPDH (accession number NM_002046) were 5' AAGGTGAAGGTCGGAGTCAACG 3' in the forward direction and 5' GGCAGAGATGATGACCCTTTTG 3' in the reverse direction. Amplification was performed using 2 µl of RT reaction product under the following conditions: 95 ºC for 4 mins, followed by 30 cycles at 95ºC for 30 s, 60 ºC for 30 s and 72 ºC for 30 s with a final extension at 72 ºC for 10 mins. As a negative control, 2 µl of the RT-PCR product synthesised in the absence of reverse transcriptase was used as a template. PCR products were electrophoresed on a 1.5 % agarose gel and the sequence of PCR products confirmed (Cytomyx, Cambridge, UK).

IP receptor antibody production

A peptide corresponding to the C terminus of the IP receptor (RRDPRAPSAVGKE) was synthesized and conjugated to hemocyanin before injection into rabbits using standard protocols (Eurogentec Seraing, Belgium). Bleeds were assayed for activity using a standard ELISA assay, and those showing reactivity were affinity purified.

Immunohistochemistry

Tissue sections: Archival tissue blocks from patients with IPAH including blocks from explanted lungs at transplantation and from patients without cardiopulmonary disease were used with the permission of the Local Ethics Committee (ICH and GOSH REC 05/Q0508/49). Blocks were processed for histology and 5 μm sections were immunostained with antibodies to α-SM actin, the endothelium (CD31, Dako UK, Ely UK), PPARγ (2492, 2435 Cell Signalling, Danvers MA, USA), and the IP receptor (see above) while Ki67 (Zymed, Invitrogen Ltd, Paisley, UK) was used as a marker of cell replication, as previously described (5). Primary antibody binding was visualised using diaminobenzidine and specificity of staining was controlled by incubation with an inappropriate secondary antibody. Slides were examined using a Leitz Dialux 20 microscope and images acquired and analysed using a Leica DM LB microscope with a Micropublishing 3.3RTV camera and QCapture Pro software (Media Cybernetics, Bethesda, MD, USA). Staining for the IP receptor and PPARγ expression was assessed semiquantitatively in all normal and IPAH cases. Slides were anonymised to ensure blinding of scoring. Apart from images shown in figure 3A, all sections were examined using x40 objective re and intra-acinar arterial sections werescored for presence or absence of positively staining cells within the medial and intimal PASMCs. Scoring was: - 0 for unlabelled, 0.5 for patchy labelling, 1 for uniform weak labelling and 2 for uniformly strong labelling of medial and intimal proliferative layers of smooth muscle and for endothelium. For each group, normal, untreated and treated IPAH, scores were analysed.

Isolated PASMCs and HEK-293 cells immunostained for the IP receptor:

Cells were cultured on 8-chambered slides (BD Biosciences, Oxford, UK) coated with 0.01% (w/v) poly-L-lysine (Sigma). All subsequent steps were done at 4°C unless otherwise stated. Chambers were washed in PBS before incubation with a 4% paraformaldehyde solution for 10 mins. After two 10 min washes with large volumes of PBS, cells were permeabilised with 0.4% Triton X-100

(Sigma) for 20 mins and then incubated with control blocking solution (2% BSA, 5% goat serum, 0.2% Triton X-100 in PBS) for 4 hr. The IP receptor antibody was diluted 1:100 in PBS and centrifuged at 10,000 g for 5 mins to remove any aggregated material. 100 µl of primary antibody was added to each well and incubated overnight in a moist chamber. As a control in some experiments, cells were pre-incubated with the control peptide (2 mg/ml) for 3 hr before incubation with primary antibody. Following four washes with PBS, antibody binding was visualized by incubation with a fluorescent secondary antibody. 100 µl of goat anti-rabbit Alexa 488 (Invitrogen), together with the nuclear stain, TO-PRO-3 (Invitrogen), were applied (1:300 dilution) and incubated for 1 hr in a moist darkened chamber at room temperature. After a final set of four washes with PBS, the slides were air dried and mounted with Vectashield (Vector Laboratories, USA). Slides were viewed and analysed at ×60 magnification (water immersion lens) using a Bio-Rad Radience 2000 laser-scanning confocal Nikon TE1000 microscope (Hemel Hempstead, UK). Green fluorescence of FITC/Alexa 488 and far red fluorescence of TO-PRO-3 were measured at an excitation/emission wavelength of 492/520nm or 644/657nm, respectively. No fluorescence was detected following incubation with either primary or secondary alone, confirming specificity of immune staining. A z-stack of 10 images was aquired representing a 0.1µm thick section through the middle of the cell using Bio-Rad LaserPix 4.0 software.

Western blotting

Protein samples were separated using a 7.5% salt SDS-PAGE and then transferred electrophoretically to nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK). Blots were washed in Tris buffered saline containing 0.1% Triton X-100 (TTBS) and blocked with 5% milk in TTBS, before being incubated overnight with the IP receptor antibody (1:500 dilution. After several washes, blots were incubated for 1hr at room temperature with donkey anti-rabbit peroxidase (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by several washes

in blocking buffer. All primary and secondary antibodies were diluted in 1% milk in TTBS. Blots were processed and developed using the ECL Plus chemiluminescent immunoblot detection system (Amersham Biosciences) and Amersham hyperfilm. For beta actin staining, blots were stripped with stripping buffer (2% SDS and 7µl/ml β-mercaptoethanol in 0.05M Tris pH 6.8) for 30 min at 55^0C and re-probed with mouse monoclonal anti-β-actin (Sigma AC15 diluted at 1:10,000) for 1 hr at room temperature to assess loading density. Protein content was measured by densitometry using NIH Image software and IP band densities, of normal and IPAH PASMC extracts, normalized to those of β-actin, within each blot were compared.

Intracellular cyclic AMP extraction and measurement

Human PASMCs were grown to 70-80% confluence in 6 well plates and starved in low serum (0.1%) media for 48 hr before being stimulated for 30 mins with media containing 10% FBS in the presence of the IP receptor agonist with or without the antagonist. The 30 min time point was chosen as we had previously determined that cAMP generation induced by treprostinil peaked at this time point, while the dose and choice of the adenylyl cyclase inhibitor (100 µM 2'5'-dideoxyadenosine) was chosen on the basis that it essentially inhibited cAMP elevation over the entire concentration-range of various prostacyclin analogues (6). Likewise the IP receptor antagonist concentration was determined by its ability to fully inhibit cAMP elevation by treprostinil in HEK-293-IP cells. Cyclic AMP was extracted by incubating cells in 0.1 M HCl for 20 min at room temperature followed by homogenisation then centrifugation at 1000 g for 10 minutes at 4°C. The protein concentration in the supernatant was determined using a Bradford based protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK). Intracellular cAMP was measured using a competitive enzyme immunoassay 96 well plate kit (Cyclic AMP ACE EIA kit, Cayman Chemical, Ann Arbor, MI, USA). The assay was performed according to the

manufacturer's instructions. Each sample was run in duplicate and data expressed as pmol of cAMP per mg of total protein.

The effect of treatments on intracellular cAMP levels in mouse cells was investigated using a radioimmunoassay (RIA). Mouse PASMCs were grown to sub-confluence (~80-90%) in 48 well plates containing DMEM and 10% FBS. Cells were then washed twice with PBS and incubated for 3 hours in serum-free DMEM (1ml/well), which represented time zero. Fresh DMEM was then added to individual wells in the absence and presence or cAMP elevating agents, which were incubated for varying times (1, 2, 4, 6, and 24 hr). Each control or treatment group comprised a minimum of 4 wells. Treatments were terminated by aspiration of medium and the addition of cell lysis buffer (acidified ethanol containing 500μM IBMX). Plates were then sealed and kept at -20^0C overnight to complete cell lysis after which 125μl was removed from each well and vacuum-dried overnight. A radioRIA for cAMP was performed using a commercially available kit (NEN^{TM}-Life Science Products Inc, Boston MA) which included [^{125}I]-labelled cAMP. Cell number counts were then performed on representative wells at various time points and final results expressed as pmol/10^6 cells for each treatment.

Luciferase reporter assay

Cells were transfected in suspension using Lipofectamine 2000 (Invitrogen). The luciferase reporter construct pGAL5TKpGL3 was transiently co-transfected into HEK-293-IP cells with the Renilla control vector, pMLuc2 and the GAL4-hPPARγ-pcDNA3 reporter construct as previously described (3). Transfected cells were seeded onto 96 well plates at a density of 1 x 10^5 cells/ml and left for 48 hr in medium containing 10% FBS. After 48 hr, cells were either left untreated or stimulated with agonist and/or antagonist at varying times for up to 24 hr. Total cell lysates were prepared using 1X passive lysis buffer (Promega, Southampton, UK). Luciferase and Renilla

activities were determined using a dual luciferase assay system (Promega) in a Tropix TR717 microplate luminometer (Applied Biosystems, Warrington, UK) according to manufacturer's instructions. Background values from untransfected cells were subtracted from all luciferase and Renilla readings. The luciferase values were normalised to Renilla values and expressed as either mean fold increase or relative light units from untreated cells.

Reagents

Treprostinil was kindly provided by United Therapeutics (Washington, MD, USA), the IP receptor antagonist RO1138452 by Roche (Palo Alto, CA, USA) and cicaprost and iloprost by Schering AG (Berlin, Germany). 2'5'-dideoxyadenosine (DDA) and GW9662 were obtained from Merck Biosciences (Nottingham, UK) and rosiglitazone from Alexis Corporation (Lausanne, Switzerland). Drugs were prepared as stock solutions in DMSO (RO1138452, rosiglitazone and GW9662) or distilled water. The final concentration of DMSO did not exceed 0.1% and this or lower concentrations had no significant effect on cell growth in any human cell line (figure 5 & 8E).

Statistical analysis

Each experiment was repeated at least 3 times and results expressed as mean±S.E.M. of n observations, except for fig.2C) where data is represented as box-whisker plots. The concentration of agent causing 50% inhibition of cell proliferation (IC_{50}) was calculated using the sigmoidal-curve fitting routine (GraphPad Software, San Diego, CA). Statistical analysis was performed using GraphPad Prism 4 with significance assessed using a Student's t-test or one-way analysis of variance (ANOVA) with correction for multiple comparisons. A P value <0.05 was considered significant.

Results

IP receptor expression in normal and IPAH PASMCs and in HEK cells

A C-terminal antibody for the IP receptor was made because we found the commercially available N-terminal Cayman antibody was non-specific. Not only did the antibody primarily stain the nuclear region of HEK-293 cells stably expressing the IP receptor (HEK-IP) or empty vector (HEK-Con) with equal effect, but there was no evidence of membrane staining in either cell line (data not shown). By contrast immunostaining with our antibody was localized around the cell margins in HEK-293-IP cells (Figure 1A) and staining barely detectable in control HEK-293 cells. Using primers to amplify a 204 bp sequence of the human IP receptor, PCR confirmed bands of the appropriate size in mRNA in HEK-293-IP cells with little IP receptor mRNA detectable in control HEK-293 cells (Fig.1B). This is consistent with previously data reporting the absence of the IP receptor in wild-type HEK cells (7). In growing normal and IPAH cells mRNA for the IP receptor were clearly evident though band intensity in IPAH cells was consistently weaker (Fig.1C). Primers for human GAPDH were used to amplify a 363 bp product of this housekeeping gene. The correct band size of similar intensity was observed in all lanes (Figure 1). Lack of bands without reverse transcriptase confirmed the absence of genomic DNA contamination in RNA samples.

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Figure legends

Figure 1. (A) Sub-confluent HEK-293 cells stained with the IP receptor antibody using FITC conjugated Alexa fluor 488 (green stain) as the secondary antibody. Images were taken under identical conditions. IP receptor mRNA levels in HEK expressing the IP receptor (HEK-IP) or empty vector (HEK-Con) (B) and in PASMCs from normal or IPAH patients (C). Lane one contains base-pair makers and subsequent numbers refer to different cell or clonal isolates. The

negative controls (absence of reverse transcriptase) were run in the last 2-3 lanes. mRNA levels for the house keeping gene are also shown in the lower panels for each cell type.

Figure 2. A-D Sections through muscular pulmonary arteries of a child with IPAH. A,B serial sections incubated with polyclonal anti IP receptor followed by incubation with (A) a biotinised anti-rabbit secondary antibody showing positive staining of medial smooth muscle (L= vessel lumen) and (B) a biotinised anti-mouse secondary as a negative control showing no detectable stain. C,D Characterisation of the arterial wall. Serial sections have been stained with (C) monoclonal anti-CD31 specific for endothelial cells and (D) with monoclonal anti-α-smooth muscle specific actin (Sigma UK clone 1A4) which stained the medial smooth muscle layers but not the endothelium (arrowed). Scale: bar represents 30μm. E Western blot of extracts of peripheral pulmonary arterial smooth muscle cells derived from 3 children with IPAH. Each lane was loaded with 7.5μg of protein and the blot stained for proteins characteristic of vascular smooth muscle; α-smooth muscle actin and smooth muscle myosin heavy chain, the β-actin band was used as a loading control.

Figure 3. Growth-arrested normal distal human PASMCs were incubated in media containing 10% FBS and either left untreated or treated with iloprost (ILO; 100nM), the IP receptor antagonist (IPRA; 1μM) or a combination. Cells were counted at 96hr following treatment. Data are expressed as % cell proliferation relative to growth response mediated by 10% FBS alone and shown as mean ± S.E.M. (n=9). *** = $P<0.001$ compared to control or as shown.

Figure 4. HEK-293 cells stably expressing the IP receptor (HEK-293-IP) were transiently transfected with GAL4-hPPARγ-pcDNA3 (expression vector for PPARγ fusion protein)

pGAL5TKpGL3 (reporter vector) and pMLUC-2 (renilla internal control). After 48 hr, cells were stimulated with 10% FBS alone (Control) or incubated with DMSO (0.1%) or the PPARγ agonist rosiglitazone (ROSI) with or without the PPARγ antagonist, GW9662, which was added 1 hr prior to the addition of ROSI. Luciferase activity was determined at 24 hr and luciferase activity normalised to renilla activity. Results are expressed as mean fold increase in luciferase activity above untreated control ± s.e.m. (n = 12, 3 separate transfections). ***= $P < 0.001$ when compared to control or as indicated.