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Protective effects of intermedin/adrenomedullin-2 in a cellular model of human pulmonary arterial hypertension

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ABSTRACT

Proliferation of pulmonary fibroblasts (PF) and distal migration of smooth muscle cells (PSM) are hallmarks of pulmonary arterial hypertension (PAH). Intermedin/adrenomedullin-2 (IMD/AM2) belongs to the Calcitonin Gene-Related Peptide (CGRP)/Adrenomedullin (AM) superfamily. These peptides act via Calcitonin-Like Receptors (CLR) combined with one of three Receptor activity-modifying proteins (RAMPs). IMD/AM2 is a potent pulmonary vasodilator in animal studies. The aim was to describe expression of IMD/AM2, AM and receptor components in human pulmonary vascular cells and to elucidate effects of IMD/AM2 on human PSM migration and PF proliferation. Gene expression was detected by immunofluorescence, immunoblotting and qRT-PCR. Normotension and hypertension were simulated by applying pulsatile mechanical stretch (Flexcell® apparatus). Viable cell numbers were determined by dye exclusion. PSM chemotaxis was measured via Dunn chamber. IMD/AM2 protein was co-expressed with AM and their receptor components in pulmonary artery and microvascular endothelial (PAEC, PMVEC) and non-endothelial cells (PF, PSM), and localised to vesicles. IMD/AM2 was secreted under basal conditions, most abundantly from PF and PMVEC. Secretion from PF and PSM was enhanced by stretch. IMD/AM2 mRNA expression increased in response to hypertensive stretch of PSM. IMD/AM2 inhibited PDGF (10^{-7} M)-mediated PSM migration maximally at 3×10^{-10} M and PF proliferation maximally at 3×10^{-9} M. Angiotensin II (5×10^{-8} M), normotensive and hypertensive stretch augmented PF proliferation. IMD/AM2 (10^{-9} M) abolished the proliferative effects of Angiotensin II and normotensive stretch and attenuated the proliferative effect of hypertensive stretch alone and combined with angiotensin II. These findings indicate an important counter-regulatory role for IMD/AM2 in PAH.

1. Introduction

Pulmonary hypertension (PH) is a rare, progressive disease characterised by a mean pulmonary arterial pressure (mPAP) greater than 25 mmHg at rest [1]. In contrast to other subtypes of PH which are secondary to another condition, Pulmonary Arterial Hypertension (PAH) has hereditary and idiopathic factors [1]. The pulmonary circulation is a system of low pressure but high flow and pulmonary arterial walls are thin. PAH is characterised by narrowing of the lumen of the small pulmonary arteries by increased vessel wall tone and thickening of the arterial wall due to vascular remodelling and proliferation, which grad-

ually increases pulmonary vascular resistance [2]. The most consistent pathological finding is the hypertrophy, proliferation and infiltration of pulmonary vascular smooth muscle cells (PSM) into the distal areas of the pulmonary tree [3]. Platelet-Derived Growth Factor (PDGF) has been implicated in PSM migration [4]. Fibroblasts have a unique role in the pathology of PAH as they are less differentiated than other cell types allowing them to rapidly migrate, proliferate, synthesise connective tissue, produce cytokines and transdifferentiate into other cell types, particularly smooth muscle cells, progressively increasing vascular tone [5]. Angiotensin II exerts a proliferative effect on pulmonary fibroblasts (PF) and contributes to vascular remodelling in PAH [6].

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Table 1
Primer sequences used in Quantitative Real-Time PCR.

Human Primer Set	Forward	Reverse
IMD/AM2	TCCAGTGAGAATCCCTACC	GAGATTGATGGCGTTGGAGG
AM	AAACTGATTCTCACGGCGTG	AATAGTGAGGCTTGGCGCC
CGRP	AACCTTAGAAGCAGCCAGGCATG	GTGGGCACAAAGTTGTCTTCACCA
Endothelin-1	GCCACCTGGACATCATTTGG	CATACGGAAACAACGTGCTCG
GAPDH	ATCGATGACAACCTTGGTATCGTG	GGCATGGACTGTGGTCATGAG
β -actin	GACTCCGGTGACGGGGTCACC	CACGATGGAGGGGCCGACTC
CLR	CATTCAACAAGCAGAAGGCG	CAGAGCCATCCATCCCAGG
RAMP1	CTGGCCCATCACCTCTTCAT	CACCGTAGTTAGCCTCTGGC
RAMP2	CGAAAAGGATTGGTGCAGCT	CAGGGTGCTATAAGGCCTGC
RAMP3	GAGGTTCTCATCCGCTGATC	CATGGCGACAGTCAGAACGA

Fig. 1. Baseline mRNA expression of the peptides (a)-(d) and their receptor components (e)-(h) in PAEC, PMVEC, PSM and PF, respectively. Data are expressed as percentage relative to GAPDH mRNA levels and are the mean values + S.E. of $n > 3$ independent cell cultures run in triplicate.

The formation of plexiform lesions found in PAH has been attributed to disorganised proliferation of pulmonary aortic endothelial cells (PAEC). Endothelial dysfunction also has implications for imbalance between vasodilators such as prostacyclin and nitric oxide and vasoconstrictors such as endothelin-1 [7]. Such vasoactive mediators have been the targets of treatments for PAH including the phosphodiesterase-5 inhibitor, sildenafil [8], prostacyclin analogue epoprostenol [9] and endothelin receptor antagonist, bosentan [10]. However, success has been limited and survival rates remain low [1].

Intermedin/adrenomedullin-2 (IMD/AM2) is a member of the calcitonin gene-related peptide (CGRP)/adrenomedullin (AM) peptide family. Association of the common calcitonin-like receptor (CLR) protein with one of three receptor activity modifying proteins (RAMPs) gives rise to CGRP-preferring (CGRP) and AM preferring (AM₁ and AM₂) receptors; IMD/AM2 displays approximately 10-fold selectivity for the AM₂ receptor relative to that for AM₁ and CGRP-preferring receptor subtypes [11]. CGRP-expressing nerves are found in the lamina propria of the normal human lung and adjacent to blood vessels, as well as in some submucosal ganglia and nerve bundles [12]. CGRP has also been detected in human alveolar epithelial cells and neonatal bronchial neuroendocrine cells [13,14]. Within the human systemic cir-

culation, AM and IMD/AM2 are expressed in aortic endothelial and smooth muscle cells [15,16] and are present in plasma [17]. AM and IMD/AM2 are found in rodent lung [18]. AM is expressed in human pulmonary endothelial cells (PEC) [19] and PSM [20]. The distribution of IMD/AM2 specifically in human pulmonary vasculature is relatively unknown. CGRP [21] and AM [22] are both elevated in plasma of PAH patients. CLR is expressed by the pulmonary microvascular endothelium [23] and within alveolar capillaries [24] and bronchial venules [25]. RAMP2 is highly expressed in lung tissue [26] suggesting a functional role for the AM₁ receptor. Within the systemic vasculature, RAMP1 is most prominent in the smooth muscle of smaller arteries and arterioles [27] whilst RAMP2 is predominantly found in human aortic endothelial cells (HAEC) and human aortic smooth muscle cells (HASMC) [28]. However the distribution of RAMPs is much broader than that of CLR, indicative that RAMPs may have additional roles in cellular physiology aside from their functional relationship with CLR [29]. The relative expression of RAMPs within the human pulmonary circulation remains largely unknown.

CGRP, AM and IMD/AM2 mediated vasodilation is generally only observed when tone is increased across the pulmonary vascular bed or after pre-contraction of isolated pulmonary vascular rings [30]. CGRP

Fig. 2. Representative immunofluorescence images: (a) IMD/AM2; (b) AM showing the peptide alone (upper panel: green) and merged immunofluorescent images (lower panel: peptide, F-actin and cell nucleus, stained green, red and blue, respectively); (c) peptide content relative to that of β -actin for AM and IMD/AM2. Difference between AM and IMD/AM2 levels * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$. Images were analysed for $n = 35$ cells obtained from $n = 15$ microscopic fields and for $n = 3-4$ independent cultures. Peptide signals have been intensified in the representative micrographs to facilitate visualisation of their cellular distribution.

inhibits PSM proliferation [31] while AM promotes sprouting in porcine pulmonary arterial endothelial cells and promotes new vessel formation in rodent [32]. The two most common rodent models, the chronic hypoxic model and the monocrotaline injury model, have contributed to understanding of the human PAH process but are not without limitations [33]. CGRP and AM have been found to attenuate pulmonary artery pressure and vascular remodelling in such models [34,35]; a vasorelaxant effect of IMD/AM2 has also recently been reported in hypoxia-induced pulmonary hypertensive rat [36]. Evidence for receptor subtype and endothelium dependence/nitric oxide involvement in these relaxant responses is conflicting [37,38]. Such models have translated poorly in their ability to reflect remodelling associated with human PAH and to predict therapeutic success for novel pharmacological strategies [39]. Human pulmonary vascular cells have potential for exploitation in this regard but to date there are relatively few reports in the literature and specifically in regard to IMD/AM2: hypoxia increases expression of IMD/AM2 in human pulmonary microvascular endothelial cells (PMVEC) while the exogenous peptide can improve barrier function in response to pressure loading [40].

Little is currently known about the role of IMD/AM2 in the pathogenesis of human PAH. The objectives therefore were to: (1) examine the relative distribution of IMD/AM2, AM and CGRP and their receptor components across human pulmonary cell types important in the pathogenesis of PAH (PSM, PF and PMVEC) under basal conditions and explore the influence of pulsatile mechanical stretch to model normotension and hypertension; (2) investigate the influence of IMD/AM2 on chemoattractant-induced PSM migration; (3) determine the influence of IMD/AM2 on PF proliferation under basal conditions and in

response to angiotensin II and in response to pulsatile mechanical stretch to model normotension and hypertension.

2. Materials and methods

2.1. Cell culture and sub-culture

PAEC, PMVEC, PSM and PF cells were obtained from ScienCell (Germany) and grown in cell type-specific sterile growth medium (GM) in a humidified incubator at 37 °C and 5 % CO₂ in T25 flasks (Iwaki, Japan). In addition to supplied supplements, media were supplemented with: 200 $\mu\text{mol.l}^{-1}$ glutamine dimer, 2 $\mu\text{g.ml}^{-1}$ amphotericin-B, 60 $\mu\text{g.ml}^{-1}$ penicillin-G and 100 $\mu\text{g.ml}^{-1}$ streptomycin sulphate (Invitrogen, UK). Medium was replaced aseptically every 3–4 days. Cells were sub-cultured every 10–14 days when ~ 80–90 % confluent. Experiments were conducted at passage 4–8 at which point cells exhibited sufficient growth rates without loss of phenotype.

2.2. Simulated hypertension

Cyclic mechanical stretch was performed *in vitro* using the Flexcell Strain Unit (FX-5000TM Tension System, 25 mm BioFlex® Loading Station™; Dunn Labortechnik GmbH, Germany). Cells were seeded onto 6-well silicone elastomer-bottomed Flexcell plates (Dunn Labortechnik) and incubated for 48 h in the presence of varying concentrations of IMD/AM2. Flexcell plates were positioned within an incubator on the loading station to which a computer-controlled vacuum unit generated a reproducible deformation of the flexible silicone base. Subsequent release of the vacuum resulted in return of the membrane to the original

Fig. 3. IIF staining for IMD/AM2 and the membrane protein syntaxin in endothelial (PAEC) and non-endothelial cell type (PSM) showed co-localisation within these cells (a): Syntaxin (Red); (b) : IMD (Green); (c): Merged image showing IMD/AM2 and syntaxin co-localisation (Orange).The cell nucleus is stained blue.

conformation. Specific regimens were programmed: cyclic stretch equating to a mean pressure of 60 mmHg (8.0 kPa) systolic and 20 mmHg (2.66 kPa) diastolic and was applied at a frequency of 1 Hz (60 cycles per minute) for the hypertensive model. Cyclic stretch equating to a mean pressure of 15 mmHg (2.0 kPa) systolic and 7.5 mmHg (1.0 kPa) diastolic and was applied at a frequency of 1 Hz for the normotensive model. Cells were exposed to this mechanical strain for 72 h. Two identically seeded Flexcell plates with the same treatment conditions were also placed in the incubator, not on the loading station, acting as un-stretched control.

2.3. siRNA transfection

Cells were incubated with siRNA duplex (10 nM) for RAMP1 (Sc-40894), RAMP2 (Sc-36378), RAMP3 (Sc-40896) and CLR (Sc-43705) or sham siRNA (non-targeted, randomised, Sc-37007) with transfection reagent in transfection medium (all Santa Cruz). Cells were then incubated for 24 h with cell-type specific GM containing the normal amount of serum and antibiotics that cells were normally cultured in before replacement with GM used for routine cell culture. Cells were used for experiments 72 h later.

2.4. Determination of viable cell number by Trypan Blue Assay

Following trypsinisation and neutralisation with Foetal calf serum (FCS), 100 μ l of the resultant cell suspension was diluted with phosphate-buffered saline (PBS) (100 μ l) containing 0.2 % Trypan blue before introduction at the edge of a haemocytometer chamber. Viable cells were counted in quadruplet over four corner squares of the haemocytometer grid.

2.5. Chemotaxis

Cells were seeded onto Type 3 thickness coverslips (20 $\times$ 22 mm, Chance Propper Ltd. U.K.) Coverslips were viewed under a light microscope to ensure cells were not more than 50 % confluent as analysis of chemotaxis requires cells to move individually; typically there were 25–60 cells attached per coverslip. The Dunn Chemotaxis chamber (Weber Scientific, Teddington, UK) is a glass slide containing two concentric circular wells that are separated by an optical bridge that is countersunk 20 μ m below the surface of the glass slide. The outer well of the chamber contained 'positive medium' i.e. the chemo-attractant Platelet-Derived Growth Factor-BB (100 ng.ml⁻¹) while the inner well contained 'negative medium' i.e. devoid of chemoattractant. Both wells were flooded with negative medium. An inverted coverslip was put over the wells with the cells facing down. After removing the excess liquid with tissue paper the coverslip was moved to the side uncovering part of the outer well. The outer well was dried by capillary action with tissue paper, and then filled with positive medium (containing the chemoattractant) resulting in a growth factor gradient from the inner to the outer well, across the optical bridge. The surface of the coverslip was then gently dried with tissue paper and melted dental wax (Chaperlin and Jacobs Ltd., NI) brushed over the edges (avoiding the optical bridge) to prevent the coverslip from drying out. A PC with an Image capture card was connected to a 'Uniblitz' shutter control device (BFI OPTILAS, UK), and a video camera (JVC, UK) linked into the optics of an Olympus IMT-2 microscope. The Dunn chamber was set on the microscope stage, which was heated using a Linkam heated stage control (MC60, Linkam Scientific Instruments Ltd., UK) at a temperature of 37.1 °C. The optical bridge of the Dunn chamber was brought into focus with the inner well at the bottom of the screen to facilitate the subsequent tracking. The Metamorph 6.1 Imaging System (Universal Imaging Corporation, West Chester, USA) software was used to

Fig. 4. Representative immunofluorescence images: (a) merged images of PMVEC (upper panel) and PF (lower panel). CLR, F-actin and the cell nucleus are stained green, red and blue respectively; (b) cellular distribution of RAMPs 1–3 (Green, left panel) and merged image showing RAMP (Green), F-actin (Red) and the cell nucleus (Blue) (right panel). (c) Receptor component protein content relative to that of β -actin protein. Images were analysed for $n = 35$ cells obtained from $n = 15$ microscopic fields and for $n = 3$ – 4 independent cultures. Receptor component protein signals have been intensified in the representative micrographs to facilitate visualisation of their cellular distribution.

Fig. 5. Representative immunoblots for AM, IMD/AM2 and their receptor components, detected in PSM and PF (a). Molecular weights of the bands of interest were estimated from molecular weight standards run in parallel. Confirmation of the specificity of the antibodies employed to detect the proteins of interest was reportedly previously [41]; in those experiments, the identity of the CLR protein (42kDa) was confirmed by comparison with immunoblots of membranes prepared from Cos7 cells transfected with hCLR or rCLR cDNAs and from sham-transfected Cos7 cells. Although an additional band corresponding to a protein of larger molecular mass (50 kDa) in transfected Cos7 cells was also present in human PSM and PF as shown, this band has also been detected in sham-transfected Cos7 cells, indicating that the antibody used might recognize a discontinuous epitope present on another unrelated protein (50 kDa). Data expressed in arbitrary units relative to α -tubulin levels and given as the mean values $\pm$ S.E. of $n = 3$ western blots (b).

set up a stack of TIFF files (images) over an acquired time-lapse interval (one image-capture every 10 min for 18 h, 109 pictures). The experiments were run in the dark. The Informatik file combine programme (TIFFKIT v3.1; Informatik Inc., Devon, UK) was used to combine the images. JCELL Windows tracking software was used to track individual cells on the sequenced images. The horizon was set at 100 μ m (above threshold for physiological movement of PSM in the absence of a gradient and corresponding to ~ 20 % of the cell's length. Using the programme Mathematica a circular histogram was developed for Rayleigh Test analysis.

2.6. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using an RNeasy Mini-Kit (Qiagen). Total RNA content was quantified using a spectrophotometer and Nanodrop Software (Version 4). Lysate was stored at -80°C pending analysis. As shown in Table 1, reported sequences for the genes investigated were used to design human-specific primers (Primer Express software, Applied Biosystems, UK). These gene-specific primers were then synthesised by Invitrogen. Specific primers and an RNA-dependent DNA polymerase were used to amplify the gene of interest in an ABI Prism 7500 sequence detection system (Applied Biosystems). Amplified cDNA was quantified by SYBR Green detection (Abgene Biotechnologies, UK). The housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference. Analysis was performed using ABI 7500 Prism software with the cycle threshold (Ct) value at which a statistically significant increase in signal occurred being used to ascertain expression level. Statistical analysis of results was performed using $\Delta\Delta$ CT (Ct gene of interest – Ct housekeeping gene). All runs were carried out in triplicate.

2.7. Protein extraction and immunoblotting

Medium was removed and cell monolayers washed x2 with ice-cold PBS prior to incubation with 350 μ l radioimmunoprecipitation Assay (RIPA) buffer (1 % vol.vol $^{-1}$ Triton X-100, 0.1 % vol.vol $^{-1}$ sodium dodecyl sulphate and protease inhibitors in PBS) for 20 min at 4°C . A cell scraper was used to detach cells from the growing surface. Lysate was transferred to a micro-centrifuge tube and placed on rotator mixer for 1 h at 4°C before centrifugation (Eppendorf Centrifuge 5417R) at 10,000 rpm for 10 min. Total protein content of the separated supernatant was quantified using a spectrophotometer and Nanodrop Software (Version 4). Aliquots were stored at -20°C pending analysis. Whole cell-lysate protein (100 μ g) reduced in equal volume of Laemmli buffer (containing 5 % vol.vol $^{-1}$ β -mercaptoethanol) was denatured (95°C for 5 min) and resolved in 4–12 % Bis-Tris gels (Invitrogen). Gels were run at 200 V for 1 h before transfer to a polyvinylidene fluoride membrane (PVDF; 30 V for 1 h). Successful transfer was verified by Coomassie blue staining of the gel. Membranes were blocked for 1 h at 4°C [20 ml; 2.5 % BSA in TBS-T (Tris-buffered saline) containing 0.1 % Tween20]. Proteins were detected using the SNAP i.d system (Millipore Corporation, USA). Solutions of primary antibody (Santa Cruz, USA) were used at a dilution of either 1:200 (RAMP1, RAMP3, CLR, AM, IMD/AM2 and α -Tubulin) or 1:1000 (RAMP2) in TBS-T. Horseradish peroxidase (HRP)-labelled anti-species secondary antibody diluted to 1:2000 in TBS-T was then added to the membrane before incubation with a chemiluminescent detection reagent (SuperSignal West Dura Extended Duration Chemiluminescent Substrate, Pierce Biotechnology, USA). Analysis was carried out using VisionWorks LS Analysis software (Ultra-Violet Products Ltd., UK).

Fig. 6. Circular histograms showing the proportion of PSM cells migrating in each direction; arrow and green wedge indicate mean direction of movement and 99 % confidence interval respectively. Representative migratory pattern of PSM in a concentration gradient of PDGF (10^{-7} M) (a): $n = 49$, $*P < 0.05$ (Rayleigh test: chemotaxis detected); IMD/AM2 (10^{-9} M) inhibited PSM migration induced by PDGF; $n = 33$ (b). Quantitative analysis of inhibition of PDGF mediated migration of PSM by IMD/AM2 (3×10^{-10} M); $n > 30$ cells from 3 independent experiments were used (c). $+++P < 0.001$ (t-Test: % Net migration vs. control). Concentration-dependence of the inhibitory effect of IMD/AM2 and attenuation of this effect by transfection with CLR siRNA (d); data are presented as means for $n = 30$ cells for each experiment. $**P < 0.01$: Tukey's multiple comparison test vs. PDGF Control. $+++P < 0.01$: Tukey's multiple comparison test vs. IMD/AM2 (3×10^{-10} M).

2.8. Immunofluorescence

After aspiration of medium, 12-well plates with coverslips containing adherent cells were washed with ice-cold PBS. Paraformaldehyde (4 %) was then added (1 ml per well) and incubated at room temperature (RT) for 15 min to fix cells. Coverslips were subsequently washed for 10 min x5 with PBS before cells were permeabilised with PBS containing 0.5 % Triton X-100 for 20 min at room temperature. Coverslips were washed 10 min x3 with PBS before blocking at room temperature for 30 min with PBS containing 1 % Bovine Serum Albumin (BSA) and 0.5 % Triton X-100 (PBS-BSA-T) to decrease non-specific binding. Primary anti-human antibodies were diluted in PBS-BSA-T at a ratio of 1:200 and used at a volume of 80 μ l per coverslip. (Human IMD1-53 primary antibody supplied by Phoenix Pharmaceuticals, CA, USA; Syntaxin and RAMP2 primary antibody from Abcam, UK; RAMP1, RAMP3 and CLR primary antibodies all Santa Cruz, USA). The specificity of the RAMP and CLR antibodies used to detect the RAMP monomers and CLR protein was confirmed previously in immunoblots of membrane samples prepared from Cos7 cells transfected with hRAMP1, hRAMP2, hRAMP3, and hCLR cDNAs (obtained from Dr. David Poyner, School of Life and Health Sciences, Aston University, Birmingham, UK) and rCLR cDNA (obtained from Prof. Walter

Born, Laboratory for Orthopaedic Research, University of Zurich, Switzerland) and sham-transfected Cos7 cells. [41].

Coverslips were subsequently incubated in a humid chamber for 1 h at room temperature before washing x3 with PBS. Anti-species fluorescein isothiocyanate (FITC; Abcam, UK) diluted to 1:500 in PBS-BSA-T was used as a secondary antibody while Alexafluor phalloidin 535 nm (Invitrogen, UK) at a ratio of 1:50 was utilised to counterstain the actin cytoskeleton. Anti-species tetramethyl rhodamine isothiocyanate (TRITC; Abcam, UK) was used as an alternative to phalloidin when cells were stained with syntaxin. Secondary antibody solution (80 μ l) was added to each coverslip before further incubation in a humid chamber for 1 h in the dark. After a further PBS wash, 300 μ l of 4', 6-diamidino-2-phenylindole (DAPI; 300 nmol.l⁻¹ PBS) was added to each coverslip preparation and incubated in darkness for 5 min in a humid chamber at RT. Coverslips were washed x3 with PBS before mounting on slides with 7 μ l Vectashield (Vector Laboratories, UK) and stored in darkness at 4 °C until examination by fluorescence microscopy (Olympus IX-51, Olympus Inc., USA.) and micrograph capture (7.3 Three Shot Color, Diagnostic Instruments Inc., USA). Spot software (version 4.1, Diagnostic Instruments) was used for picture processing and semi-quantitative analysis. Standard settings for gain and exposure were used for each cell type with no image intensification. Semi-quantitative analysis measured the ratio of fluorescent signal (green) emitted from the secondary antibody under investigation to the equiv-

Fig. 7. Concentration-dependent anti-proliferative effects of IMD/AM2, AM and CGRP on PF after 48 h treatment (a); cell counts are normalised to that in the absence of peptide (Control). Data are presented as means + S.E. for $n = 3-6$ experiments. * $P < 0.05$; ** $P < 0.01$; Tukey's multiple comparison test vs. Control. Anti-proliferative effect of IMD/AM2 on PF after 48 h treatment in combination with Angiotensin II (5×10^{-8} M) (b); cell counts are normalised to that of the Control (without Angiotensin II or IMD/AM2). Data are presented as means + S.E. for $n = 6$ experiments. ** $P < 0.01$; Tukey's multiple comparison test Angiotensin II vs. Control; # $P < 0.05$; Tukey's multiple comparison test IMD/AM2 vs. absence of IMD/AM2. Anti-proliferative effect on PF after 48 h treatment with IMD/AM2 (10^{-9} M) after knockdown of CLR or individual RAMPs (c); cell counts are normalised to that of the Control (No IMD/AM2 or transfection). Sham: randomised nucleotide sequence siRNA transfection. Data are presented with as means + S.E. for $n = 3$ experiments. ** $P < 0.01$; Tukey's multiple comparison test vs. Control.

alent (red) from the cytoskeletal antibody. The size-matched background fluorescent signal was subtracted in both cases to calculate the true fluorescent mean. Data were screened for outliers by excluding values greater or less than the mean ratio ± 3 S.D.

2.9. Quantification of IMD/AM2 content in culture media

Samples taken from media of cultured cells were acidified with an equal volume of trifluoroacetic acid (TFA, 1 % vol.vol⁻¹, Sigma-Aldrich, UK and centrifuged at $12,000 \times g$ for 20 min at 4°C. The supernatant was applied onto pre-equilibrated C18 SEP columns Waters Corporation, UK eluted with 60 % acetonitrile (Riedel de Haen, Germany) in 1 % TFA and evaporated to dryness using a combination of centrifugal concentration (Hetero Vac, Scandinavia) and lyophilisation (Edwards, Modalyo). Samples were reconstituted and assayed using a commercial ELISA (Phoenix Pharmaceuticals Inc, Karlsruhe, Germany).

2.10. Statistical analysis

Results were analysed in GraphPad Prism v. 5 (GraphPad Software Inc., Ca, USA). Where appropriate, variation within groups was assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Unpaired t -test was used when comparing two columns.

3. Results

3.1. Expression of CGRP family peptides and their receptor components

At mRNA level, AM was the most abundantly expressed peptide in both PMVEC and non-endothelial cells but IMD/AM2 was most highly expressed in PAEC (Fig. 1). The highest levels of mRNA expression of AM and IMD/AM2 were found in PSM and PAEC. IMD/AM2 and CGRP mRNAs were present but at the limits of detection in PMVEC and PF. CLR and RAMP(1-3) were also expressed across all cell types but at very low levels in PMVEC and PF. At protein level, expression of both IMD/AM2 and AM was detected in all four cell types by immunofluorescence. Semi-quantitative analysis indicated that AM was most abundant in PSM, while IMD/AM2 was more abundant in PSM and PMVEC than PF and PAEC. Representative immunofluorescence images and semi-quantitative analysis are shown in Fig. 2. IMD/AM2 was co-localised with the vesicular protein, syntaxin, in both endothelial and non-endothelial cells (as evidenced in experiments conducted in PAEC and PSM), indicating that intracellular storage of the peptide is within vesicular structures (Fig. 3). CLR and each of the RAMPs were also detected by immunofluorescence in all four cell types. RAMP2 was particularly abundant in PMVEC, and RAMP3 in PF. Representative images are depicted in Fig. 4 (a and b) and semi-quantitative analysis in

Fig. 8. Anti-proliferative effect of IMD/AM2 (10^{-9} M) on PF during 72h stretch at pressures equivalent to 15 mmHg systolic and 7.5 mmHg diastolic to simulate normotension (a). Cell counts are normalised to that of growth medium (GM) control. Data are presented as means + S.E. $n = 3$ experiments. * $P < 0.05$: GM stretched vs. unstretched control. # $P < 0.05$: IMD/AM2 vs. GM stretched. Anti-proliferative effect of IMD/AM2 (10^{-9} M) on PF after 72h stretch at pressures equivalent to 60 mmHg systolic and 20 mmHg diastolic to simulate hypertension in the absence and presence of angiotensin II (5×10^{-8} M) (b); cell counts are normalised to that of GM Control. Data are presented with as means + S.E. for $n = 4$ experiments. * $P < 0.05$: Tukey's multiple comparison test vs. Angiotensin II Flexed. Anti-proliferative effect of IMD/AM2 (10^{-9} M) on PF after 72h hypertensive stretch following siRNA knock-down of CLR and individual RAMPs (c). Cell counts are normalised to that of GM Control. Data are presented with as means + S.E. for $n = 3$ experiments. * $P < 0.05$; ** $P < 0.01$: Tukey's multiple comparison test vs. GM.

Fig. 4(c). Protein expression was confirmed by immunoblotting. Representative blots and quantitative analysis for PF and PSM are shown in Fig. 5.

3.2. Influence of IMD/AM2 on PSM migration

PDGF 10^{-7} M induced migration of PSM (46 % net migration, $P < 0.01$). IMD/AM2 inhibited PDGF-induced migration ($P < 0.01$); maximum inhibition was observed at 3×10^{-10} M (5 % net migration). Representative Rayleigh plots and quantitative analyses are shown in Fig. 6. The inhibitory effect of IMD/AM2 was attenuated (68 %, $P < 0.01$) by siRNA knockdown of CLR.

3.3. Influence of angiotensin II and IMD/AM2 on PF proliferation

IMD/AM2 decreased the basal proliferation of PF over a 48 h period. Maximum attenuation of 35 % was observed at 3×10^{-9} M ($P < 0.01$). For comparison, maximum attenuation by AM was observed at 10^{-8} M (21 %) and by CGRP at 10^{-9} M (13 %) (Fig. 7a). Angiotensin II (5×10^{-8} M) increased PF cell number by 69 % ($P < 0.01$) after 48h. IMD/AM2 attenuated angiotensin II stimulated proliferation;

the proliferation response to angiotensin II was abolished by IMD/AM2 $> 3 \times 10^{-9}$ M (Fig. 7b). The anti-proliferative effect of IMD/AM2 was inhibited ($P < 0.01$) by siRNA knockdown of CLR or to a lesser extent siRNA knockdown of RAMP2; some attenuation by siRNA knockdown of RAMP3 was observed but was not statistically significant, while siRNA knockdown of RAMP1 was ineffective (Fig. 7c).

3.4. Influence of cyclic mechanical stretch on PF proliferation

After 72h of cyclic mechanical stretch at pressures equivalent to 15 mmHg systolic and 7.5 mmHg diastolic similar to that experienced in the normotensive pulmonary artery, PF cell number was 41 % greater than un-stretched control (Fig. 8a). Treatment with IMD/AM2 10^{-9} M during stretch abolished the increase in cell number compared to that of cells stretched in the absence of peptide ($P < 0.05$). After 72h of stretch at pressures equivalent to 60 mmHg systolic and 20 mmHg diastolic to simulate hypertension, stretch alone increased cell count by 54 % relative to control. Addition of angiotensin II (5×10^{-8} M) together with stretch increased PF cell count by 116 % relative to control (Fig. 8b). IMD/AM2 10^{-9} M attenuated PF proliferation in response to hypertensive stretch (by 26 %, $P < 0.05$) and an-

Fig. 9. Viable PSM cells attached to Matrigel after 72h stretch to stimulate hypertension in the absence and presence of IMD/AM2 (2×10^{-11} M). Data are expressed as a percentage of that of un-stretched cultures incubated in the absence of IMD/AM2 and are the means $\pm$ S.E. for $n = 4$ experiments. (Fig. 9). *** $P < 0.0001$ vs un-stretched cells; + $P < 0.05$; ++ $P < 0.01$ with vs without IMD/AM2: Tukey's multiple comparison test.

giotensin II and hypertensive stretch together (by 30 %, $P < 0.05$). The inhibitory effect of IMD/AM2 was attenuated by siRNA knockdown of CLR ($P < 0.01$) and to a lesser extent by siRNA knockdown of RAMP2 ($P < 0.05$); siRNA knockdown of RAMP3, but not RAMP1, also tended to reduce the inhibitory effect of IMD/AM2, but the attenuation achieved was not statistically significant (Fig. 8c).

3.5. Influence of cyclic mechanical stretch on PSM viability and peptide expression

Stretch to simulate hypertension resulted in a marked loss of attached viable PSM cells on Matrigel at 72 h (cell viability 13.0 ± 1.9 % relative to un-stretched control, mean $\pm$ S.E. $n = 4$, $P < 0.001$) (Fig. 9). IMD/AM2 (2×10^{-11} M) enhanced attached viable cell number in both stretched (33.3 ± 9.2 %, mean $\pm$ S.E., $n = 4$, $P < 0.01$) and un-stretched PSM (120.4 ± 3.7 %, $n = 4$, $P < 0.05$).

Endothelin ET-1 mRNA expression in PSM followed a biphasic response to stretch to simulate hypertension, with marked but transient up-regulation at 48 h (19.2-fold; $P < 0.0001$) after which time expression returned to control levels at 72 h (Fig. 10a). Expression of IMD/AM2 mRNA was increased relative to control both at 48 h (1.6-fold) and 72 h (2-fold; $P < 0.01$) in response to stretch to simulate hypertension (Fig. 10b). Expression of CLR (10.1 fold, $P < 0.05$), RAMP1 (10.6 fold, $P < 0.05$) and RAMP2 (10.7 fold, $P < 0.01$), but not RAMP3 mRNA, was transiently increased at 48 h by stretch but returned towards control levels thereafter.

3.6. Influence of cyclic mechanical stretch on IMD/AM2 secretion in culture

IMD/AM2 was secreted into the culture medium by un-stretched PF (6.98 ± 2.01 ng.ml $^{-1}$) and PMVEC (12.63 ± 1.38 ng.ml $^{-1}$); in contrast there was little secretion from un-stretched PSM (0.40 ± 0.06 ng.ml $^{-1}$) and PAEC (1.28 ± 0.24 , ng.ml $^{-1}$); data are mean $\pm$ S.E. of > 4 replicates (Fig. 11). Stretch to simulate normotensive conditions enhanced secretion of IMD/AM2 from PSM (267 fold, $P < 0.05$) and PF (7.4 fold, $P < 0.05$) but not from PMVEC (1.1 fold). In contrast, stretch to simulate hypertensive conditions enhanced secretion of IMD/AM2 from PMVEC (1.4 fold vs normotensive stretch, 1.5 fold vs un-stretched, $P < 0.05$) and also enhanced secretion of the peptide from PSM (209 fold, $P < 0.05$) and PF (6.0 fold, $P < 0.05$) compared to un-stretched cells, but a lesser increase than that observed under normotensive conditions. Stretch did not enhance secretion from PAEC under normotensive (0.48 ± 0.06 , ng.ml $^{-1}$) or hypertensive (0.82 ± 0.07 ng.ml $^{-1}$) conditions.

4. Discussion

4.1. Cellular distribution of the IMD/AM2, AM and their receptor components in the human pulmonary vasculature

This study has extensively characterised expression of IMD/AM2 across a range of human pulmonary vascular cell types, and enabled direct comparison with that of AM and their shared receptor components, at both mRNA and protein level. Since each cell type was cultured in isolation, and both peptides were detected at mRNA level as

Fig. 10. Temporal dependence of the effect of simulated hypertension on mRNA expression in PSM; (a) ET-1 and IMD/AM2; (b) receptor components. Data are expressed relative to β -Actin mRNA levels and are the mean values $\pm$ S.E. of $n = 2$ experiments in triplicate. Individual replicates were used to generate error bars. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: Tukey's Multiple Comparison test vs. control.

Fig. 11. IMD/AM2 content of culture medium of un-stretched cells and in response to normotensive and hypertensive stretch. Data are given as ng.ml⁻¹ and are the mean + S.E. of > 4 replicates. *P < 0.05 difference relative to un-stretched cell; *P < 0.05 difference between hypertensive and normotensive stretch: Tukey's Multiple Comparison test.

well as protein level, the findings are consistent with their synthesis rather than uptake as an endocrine or paracrine mediator secreted by another cell type. In this regard, the work is novel since previous studies had been largely limited to lung tissue homogenate and non-human species [18,40]. IMD/AM2 was co-expressed with AM at mRNA and protein level in both human pulmonary endothelial and non-endothelial cells. This is consistent with previously reported AM expression in human pulmonary endothelial cells [19] and PSM [20] and also with that of IMD/AM2 in both endothelial and non-endothelial cell types within the human heart [16]. Very low levels of CGRP mRNA expression were also detected, most evidently in PSM and consistent with previous observations that expression of this neuropeptide is not in fact exclusively neuronal, since CGRP has been detected in human alveolar epithelial cells [42] and in lymphocytes residing in the gastric mucosa [43].

Although protein levels of AM were similar to those of IMD/AM2 in PF and PSM, they were greater in PAEC and less in PMVEC, suggesting a shift in relative influence of the two peptides from macro- to microvasculature. This is consistent with the much greater levels of IMD/AM2 detected in culture media of PMVEC than PAEC. Discordance between IMD/AM2 mRNA and protein expressional data might reflect low rates of transcription and extensive cellular storage of the translated peptide under physiological conditions. Levels of IMD/AM2 protein were similar in PSM and PF, although basal peptide levels were much higher in culture media from the latter, suggesting that secretion from PF might represent an important source of paracrine IMD/AM2 under resting conditions. High IMD/AM2 content in PSM, despite relatively little secretion, is consistent with extensive vesicular reserve in the absence of cyclic mechanical stretch, as evidenced by localisation with syntaxin.

CLR and RAMP1–3 were detected in all cell types studied at mRNA and protein level, consistent with a potential role for IMD/AM2 and other members of the peptide family as paracrine mediators produced in one cell type and exerting effects on another in the human pulmonary vasculature. CLR expression has been reported previously in microvascular endothelium from various circulations, including pulmonary [23] while RAMP2 was the most abundant RAMP detected at protein level in rat lung homogenate [44]. Co-expression of CLR and RAMPs within each cell type studied is consistent with a physiological

influence of the peptide family at functional CGRP, AM₁ and AM₂ receptors within the pulmonary circulation. Some of each RAMP protein was localised to the nucleus, inferring a nuclear function. Indeed additional roles for RAMPs in cellular physiology aside from their relationship with CLR have been proposed [29]; predominant involvement of a particular CGRP/AM receptor subtype cannot necessarily be implied from cellular abundance of the associated RAMP.

4.2. Simulated normotension and hypertension

Vascular cells, depending on their location within the vessel wall, are exposed to both fluid shear stress as a consequence of blood flow and periodic stretch and relaxation due to oscillations in vessel diameter associated with pulsation [45]. Shear stress is predicted to exert a greater influence on the endothelium. Pulsation is more relevant to PSM within the tunica media and is simulated by the model employed in the present study [46]. The flexcell apparatus was configured to reproduce arterial pulsation at a frequency analogous to normal heart rate and rhythm. Uniform equibiaxial mechanical strain was applied at two settings, equivalent to normotension and hypertension, respectively. In addition to significant reduction in viable cell number in response to hypertensive stress, a further limitation of such models is the risk of detachment of growing cells from covalently bound matrix surfaces such as collagen or laminin in mechanically active environments [46]. Prior coating of wells with Matrigel provided an adequate attachment surface for PSM. This substitute basement membrane contains a similar protein distribution to that present *in vivo*, its major components being laminin, collagen type IV, heparin sulphate and entactin [47]. Indeed IMD/AM2 increased attached viable PSM cell number; this protective effect was observed at physiologically-relevant concentrations. Cytoprotective effects of IMD/AM2 have been demonstrated previously in other vascular cells in response to various stressors (for example [15,16].)

Marked upregulation of ET-1 expression in response to hypertensive stretch provides validation of the PAH model. Although endothelial cells are a major source of ET-1, PSM can also release this peptide [48]. As well as inducing chronic vasoconstriction, ET-1 drives the vascular remodelling which characterises the pathogenesis of PAH as ET-1 stimulates proliferation of PSM in an autocrine loop via ET_A receptors [49]. Consistent with up-regulation of ET-1 in this model, post-mortem studies have found prepro-ET-1 mRNA to be upregulated in PSM from PAH patients while similar findings characterise existing rodent hypoxic models of PAH [48–50]. Interestingly, after the initial peak at 48 h simulated hypertension, prepro-ET-1 mRNA expression decreased. Similar transient increases in prepro-ET-1 mRNA have been found over a 24 h period following serum-induced PSM proliferation [48]. Transient expression may be attributable to short half-life of prepro-ET-1 mRNA or activation of a counter-regulatory negative feedback influence on gene expression [51]. The temporal pattern of expression suggests that ET-1 may act as an early-response gene to simulated hypertension and is consistent with evidence that stimuli including mechanical stress may induce transcription, translation and release of the ET-1 peptide within minutes [52]. A similar temporal relationship was detected for CLR and RAMPs 1 and 2, indicative of a co-ordinated influence on the components of CGRP/AM receptor expression. Upregulation of the expression of IMD/AM2 and the receptor components CLR, RAMP1 and RAMP2 is consistent with a counter-regulatory role for the peptide and is compatible with the observed increased secretion of the peptide into the culture media of stretched cells.

4.3. Anti-migratory effect of IMD/AM2 on PSM

An important pathological feature of PAH is the directed infiltration and migration of PSM distally into the smaller arterial vessels; PDGF

has been implicated in this process and shown to induce directional chemotaxis in the Dunn chemotaxis chamber [4]. The present study provides evidence for abolition of PDGF-induced chemotaxis by IMD/AM₂, consistent with a protective, counter-regulatory role for IMD/AM₂ during the pathophysiological progression of PAH. Maximum attenuation occurred at 3×10^{-10} M; furthermore a partial reversal was observed above that concentration. Given the greater affinity of human IMD/AM₂ for AM₂ relative to AM₁ or CGRP receptors as evidenced in studies in molecularly-defined Cos7 cells [53], the inhibitory response is compatible with an action mediated predominantly via the AM₂ receptor at picomolar concentrations, consistent with the levels secreted in the culture medium by the various cell types under resting conditions. Reversal of the attenuation could be accounted for by greater occupancy of other receptor subtypes at increasing nanomolar concentrations.

4.4. Anti-proliferative effect of IMD/AM₂ on PF

This study is the first to characterise the effect of IMD/AM₂ on PF proliferation. IMD/AM₂ attenuated PF proliferation under basal conditions and in response to angiotensin II, a known proliferative stimulus [6]; maximum attenuation was observed in the range 1–10 nM (an order of magnitude greater than for the inhibitory influence of IMD/AM₂ on PSM migration). A similar inhibitory influence of IMD/AM₂ was demonstrated in PF stretched at normotensive pressures and at hypertensive pressures, alone and in combination with angiotensin II. An anti-proliferative effect of IMD/AM₂ has been demonstrated previously in cultured rat cardiac fibroblasts [54]. In the present study, the inhibitory response was markedly attenuated by silencing CLR and RAMP2 expression within PF, supporting a predominant involvement of AM₁ receptors, although a modest contribution of AM₂ receptors to the anti-proliferative effect cannot be discounted.

4.5. Conclusion

Traditional animal models inaccurately reflect the pathophysiology of PAH while mortality remains stubbornly high despite available therapies [1]. The present study has developed and validated an alternative model of PAH, based on application of pulsatile mechanical stretch [45], and employed this to investigate the influence of a novel mediator on the disease process. IMD/AM₂ was expressed in human pulmonary vascular and non-vascular cells, together with AM and their shared receptor components, and secreted under basal conditions into culture media, in large amounts particularly by PF and PMVEC, indicative of a potential paracrine role. Furthermore, IMD/AM₂ was stored abundantly in vesicles within PSM and mechanical stretch upregulated expression of IMD/AM₂ and its receptor components, and greatly augmented basal secretion of the peptide from PSM, and further enhanced that from PF and PMVEC also. Taken together with demonstration of the ability of IMD/AM₂ to prevent PSM migration and attenuate PF proliferation at physiologically-relevant concentrations, these findings point to a potentially important counter-regulatory role for this peptide in PAH which could potentially be exploited therapeutically.

CRediT authorship contribution statement

David Holmes: Formal analysis, Investigation, Data curation. **Michael Corr:** Formal analysis, Investigation, Data curation. **Gavin Thomas:** Investigation. **Mark Harbinson:** Conceptualization, Funding acquisition, Supervision. **Malcolm Campbell:** Data curation, Formal analysis, Methodology, Project administration, Supervision. **Paul Spiers:** Investigation, Formal analysis. **David Bell:** Conceptualization, Formal analysis, Supervision.

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