Differential IL-1 signalling induced by BMPR2 deficiency drives pulmonary vascular remodelling

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Abstract

Background: Bone morphogenetic protein receptor type 2 (BMPR2) mutations are present in patients with heritable and idiopathic pulmonary arterial hypertension (PAH). Circulating levels of Interleukin-1 (IL-1) are raised in patients and animal models. Whether interplay between BMP and IL-1 signalling can explain the local manifestation of PAH in the lung remains unclear. Methods: Cell culture, siRNA and mRNA microarray analysis of RNA isolated from human Pulmonary artery (PASMC) and Aortic (AoSMC) smooth muscle cells were used. R899X+/- BMPR2 transgenic mice fed western diet for six weeks were given daily injections of IL-1ß prior to assessment for PAH and tissue collection. Results: PASMC have reduced inflammatory activation in response to IL-1ß compared with AoSMCs, however PASMC with reduced BMPR2 demonstrated an exaggerated response. Mice treated with IL-1ß had higher white blood cell counts, and significantly raised serum protein levels of IL-6 and OPG plasma levels recapitulating in vitro data. Phenotypically, IL-1ß treated mice demonstrated increased pulmonary vascular remodelling. Conclusions: IL-1ß induces an exaggerated pulmonary artery specific transcriptomic inflammatory response when BMPR2 signalling is reduced.
Pulmonary arterial hypertension (PAH) is driven by vasoconstriction, inflammatory cell infiltration, vascular cell migration, proliferation and apoptosis. Molecular mechanisms regulating PAH pathogenesis are multifactorial involving potassium channels, genetic mutations, serotonin imbalances, oestradiol changes and inflammatory alterations amongst others.

Vascular remodelling in PAH is confined to the lung and patients with the disease do not exhibit alterations in systemic blood pressure or peripheral vascular pathology. This observation indicates that inherent differences in the cellular behaviour in the vascular beds could potentially be one of the possible reasons why global BMPR2 changes only lead to pathogenic remodelling of the pulmonary vasculature whilst the systemic vessels remain unaltered.

BMPR2 mutations are the primary genetic risk for PAH implicated in over 70% of heritable and 25% of sporadic cases, however disease penetrance even within families is low (around 15%). Inflammatory diseases are associated with PAH, and levels of the pro-inflammatory cytokine interleukins (IL)1/6 are increased in PAH. IL-6 over-expression in vascular cells is sufficient to induce pulmonary vessel remodelling and PAH in a rodent model. Inflammation has been shown to be increased in patients and a link with BMPR2 deficiency has been considered. Previous animal studies have demonstrated that monocrotaline (MCT) induced PAH is prevented by administration of IL-1 receptor antagonist and that Apolipoprotein-E knockout mice develop PAH in an IL-1 dependent manner, and via a lung specific putative IL-1R1 receptor. Interestingly, recent data demonstrated that mice with a heterozygous mutation in BMPR2 have an increased inflammatory response to Lipopolysaccharide. Further recent literature indicates that the IL1R1/MyD88 signalling pathway is critical to development of animal models of PAH. We therefore hypothesised that under conditions of reduced BMPR2 expression/signalling, IL-1β can act as a pulmonary specific disease
modifying secondary stimulus, thereby demonstrating a direct link between IL-1β, BMPR2 and
disease pathogenesis.

Methods

Smooth muscle cell culture and transfection

Human pulmonary artery (hPASMC) (Lonza CC-2581) from a total of 3 commercial donors and
four primary donors including those with and without disease related BMPR2 mutations and
human aortic smooth muscle cells (hAoSMC) (Lonza CC-2571) from a total of 2 donors were
maintained in smooth muscle cell media SmGM-2 including bullet kit supplements (Lonza CC-3182).
All experiments were performed between passages 4 and 8. Quiescent media (Dulbecco’s modified
eagles medium (Lonza 12-604) with 0.2% (v/v) foetal bovine serum ((FBS) Lonza 14-401) was added
for 48 hours prior to transfection with siRNA/reporter plasmid (Qiagen Cignal NFkB luciferase
reporter) and/or 6 hour stimulation with IL-1β at 10 ng/ml or BMP4 at 20 ng/ml. 13,14

RNA extraction

RNA was isolated using Trizol, Direct-zol RNA mini prep kits (Zymo research R2050) and Zymospin
column as per manufacturer’s instructions. Eluted RNA quality was assessed using the Agilent
Bioanalyzer 2100.

Microarray and Bioinformatic Analysis
3-sample RNA pools were run per sub-array with three individual RNA pool sub-arrays per condition. Agilent microarray was performed (Agilent 5190-2305, 5188-5282, 5188-5242, 5188-5327 and RNeasy mini kit, Qiagen 74104) following the Labelling protocol (version 6.6, September 2012 Agilent technologies, G4140-90040) per manufacturer’s instructions and scanned using the Agilent G2565BA scanner.

Data analysis was performed using the Bioconductor package Limma in the programming language “R”. Targets files were created containing Agilent G2565BA microarray scanner output files. Background correction, cyclic loess normalisation and averaging of repeats was performed. A linear modelling matrix was built and fitted. Gene lists were filtered discarding those unaltered by IL-1ß in the order of a log2 fold change of < 1 and for an adjusted p value of < 0.05. A pathway analysis functional output was obtained using Signalling Pathway Impact Analysis (SPIA) in R. All as described in previous papers from our group. A 2-dimensional projection of the microarray expression data was generated using the non-parametric dimensionality reduction. This was achieved using the t-distributed stochastic neighbor embedding (t-SNE) algorithm in the R package Rtse. The resulting t-SNE output was plotted with R package ggplot2. The array data will be deposited in NCBI’s Gene Expression Omnibus.

Luciferase reporter assay

Following 48 hours incubation with siRNA, reporter plasmid and stimulants, cells were lysed and firefly and renila luciferase was read using Promega dual glo assay as per manufacturers instruction and read using the Varioskan Plate reader.
Real-Time Polymerase Chain Reaction of Cellular mRNA samples

RNA (n=9-17 for each condition) was reverse transcribed to cDNA using RNA to cDNA kit (Applied Biosystems 4387406). TaqMan probes for BMPR2 Hs00176148, IL-6 Hs1075666, SOD2 Hs00167309, OPG Hs00917067, VIPR1 Hs00910453 were purchased from Thermo fisher and run in duplicate. Human ribosomal 18S Hs99999901 was used as control. Relative Quantity was calculated using the ΔΔCt method.  

Animal models

Rosa26-rtTA2×TetO7-Bmpr2R899X mice called Rosa26-Bmpr2R899X were used with mutant expression induced by doxycycline as previously described.  
16 24 x Rosa26-Bmpr2R899X transgenic mice and 12 x C57 wild-type littermates were fed western diet for six weeks and injected with IL-1β or placebo i.p. once daily for the final four weeks. Mice were assessed for inflammatory activation and PAH phenotype as previously described.  
17 Following 6 weeks of treatment the mice were given injectable anaesthesia for terminal surgery. Animals underwent full haemodynamic phenotyping including echocardiography, right and left heart catheterisation, blood sampling by cardiac puncture and a full range of tissues taken and snap frozen. All animal studies were pre-approved by Vanderbilt University Institutional Animal Care and Use Committee.

Enzyme Linked ImmunoSorbent Assay

Mouse serum samples were run using assay DY805 (OPG) and DY206 (IL-6) as per manufacturer’s instructions.
Results

IL-1β stimulation and BMPR2 dysfunction elicit vascular bed specific transcriptional regulation in smooth muscle cells

PAH is a pulmonary arterial specific disease suggesting that there may be vascular bed specific transcriptional regulation. A major portion of the vascular disease pathology within the lesions is driven by the proliferation and migration of alpha smooth muscle actin (SMA) positive cells and we therefore sought to characterize, and compare the cellular signalling profile in smooth muscle actin positive cells from the pulmonary and aortic smooth muscle cells (PASMC and AoSMC). 1235 genes were significantly differentially expressed across both cell types in response to IL-1β, 444 in PASMC contrasting with 919 in AoSMCs. Of these genes 128 overlap in both cell types (Figure 1A).

Subsequent Signalling Pathway Impact Analysis (SPIA) identified significant differences in the pathways represented by the genes specific to each cell type (SPIA graphs and tables for the genes specific to PA and Ao as supplemental Figure 1A and B respectively). Comparison of the altered PASMC pathways highlighted differences in disease relevant pro-migratory and pro-proliferative pathways. “Pathways in Cancer” and infectious disease pathways were altered containing disease relevant wnt, FADD and MEK signalling. These pathways were activated in the AoSMC cells but responses were either inhibited or suppressed in the PASMC (figure 1B).

Microarray validation using repeat and separate donor cells was performed by quantitative reverse transcription-PCR on selected pathway and disease relevant genes. Aortic smooth muscle cells
demonstrated greater increases in inflammatory genes than PASMC given the same stimulus. All genes analysed validated array findings in terms of increase or decrease of gene expression although the degree of alteration varied between platforms. Inflammatory and pro-apoptotic genes including IL-6 and OPG (Osteoprotegerin) are increased in AoSMCs to a larger extent than PASMCs. However, PASMCs demonstrated an increased baseline level of VIPR1 compared to AoSMCs but lost of expression when stimulated with IL-1β. Disease relevant receptors were also measured. There were no changes to IL-1β in PDGF receptors a or b (figure 1C).

**BMPR2 deficiency exaggerates inflammatory activation to IL-1β stimulation in PASMC but not AoSMC**

The BMP and IL-1β signalling pathways are linked through the co-localisation and co-expression of gene family members (http://www.genemania.com, supplemental figure 2) although the direct influence of one upon the other in PASMC is unknown. We sought to determine the effect of reduced BMPR2 expression in PASMCs using siRNA, and alterations induced by subsequent stimulation with IL-1β, using whole genome microarray. Knock-down of BMPR2 was confirmed by rtPCR (figure 2A). Stimulation with IL-1β resulted in the differential expression of 825 genes on the background of reduced BMPR2 expression compared to 524 in control non-targeting (Ntsi) siRNA treated cells (figure 2B). SPIA analysis highlighted changes in pathway activation in “pathways in cancer” and “rheumatoid arthritis” (SPIA graphs and tables for the genes specific to PA with and without functional BMPR2 as supplemental Figure 2a and b respectively). Overall there were significant increases in the pro-inflammatory, pro-proliferative and migratory pathways activated most notably the massive increased activation of the cytokine and chemokine signalling pathways following IL-1β stimulation in PASMCs where BMPR2 expression is reduced (figure 2C). **This is reflected in the t-SNE analysis**
showing that the microarray data clusters into the conditions. In non-targeting silencing RNA treated PASMCs the IL-1β makes an alteration in clustering however this alteration is exaggerated in the presence on BMPR2 silencing RNA (figure 2D).

For further evidence of interactions between the 2 pathways NFkB reporter plasmid (Qiagen Cignal NFkB luc reporter assay) was used to assess the changes in IL-1β signaling through NFkB in the presence and absence of BMP pathway activation. These experiments showed that activation of the BMP signaling pathway by stimulation with BMP4 repressed IL-1β stimulation of NFkB, however upon reduction of BMP signaling through the addition of silencing RNA to BMPR2 this NFkB signaling was restored (figure 2E).

To consider the effects of reduced BMPR2 expression in PASMC, validation was performed on PASMC with and without transfection using silencing RNA to BMPR2. A small panel of genes was put together from a disease relevant gene list to validate the microarray data that would cover the range expression changes observed. Minimal transcriptional changes were noted in the mock transfection samples (data not shown) however reduced BMPR2 expression combined with IL-1β stimulation caused a further increase in expression of the pro-inflammatory genes within the panel. This is not the case in AoSMC with many genes being decreased upon reduced BMPR2 expression, a summary of all the taqman carried out has been included (figure 3A).

At the individual gene level, IL-1β stimulation in conditions of reduced BMPR2 expression in PASMC resulted in a greater induction of IL-6 compared to normal BMPR2 expression in PASMC. Using the same regime, there was no change in IL-6 in Aortic cells or in PASMC taken from donors with and without known BMPR2 mutations (figure 3B). Loss of BMPR2 also caused large increases in the
expression of OPG regardless of IL-1 β stimulation in PASMC but gave rise to a decrease of OPG in unstimulated Aortic cells which was reversed by IL-1β stimulation, in donor cells increases were seen in OPG to IL-1β stimulation regardless of their mutation status (figure 3C). VIPR1 in aortic cells is unaffected by loss of BMPR2 function however PASMC showed a significant increase at baseline which was normalised by IL-1β. PASMCs taken from donors display the same pattern as commercial PASMCs where loss of BMPR2 function induces a large increase in VIPR1 expression which can be normalised to an extent by IL-1β (figure 3D).

Array validation by qPCR of receptor expression showed that expression of PDGF receptors in PAMSC increased to loss of BMPR2 expression but normalised to IL-1β stimulation, this trend is also the case in donor PAMSCs in PDGFRA, whereas PDGFRB is increased in mutant patient cells regardless of IL-1β stimulation. In AoSMC, however, loss of BMPR2 induces loss of PDGFRa and b expression (figure 3E and F).

BMPR2 deficiency exaggerates inflammatory activation to IL-1β stimulation in R899X+/− BMPR2 transgenic mice fed high fat diet

To determine if these in vitro observations were relevant in vivo we next investigated whether IL-1β supplementation alters PAH phenotype in BMPR2 mutant mouse overexpressing the mutation R899X in the BMPR2 gene. These mice have been previously shown to have disrupted BMPR2 signaling. In accordance with the protocol outlined in figure 4A, Rosa26-Bmpr2R899X were given IL-1β treatment. RVSP was unaffected however pulmonary vascular resistance (PVR) was increased in mutant mice treated with IL-1β whilst systemic blood pressure was unaffected (figure 4B). Modest increases in small pulmonary arteriole muscularisation and SMA and PCNA positive
cells (figure 4C) suggesting more advanced pulmonary vascular remodelling were also seen. Delivery of IL-1β was evidenced by the increased WBC counts. Serum levels of IL-6 were increased in mutant IL-1β treated Ros26-Bmpr2R899X animals. OPG was increased in response to IL-1β regardless of mutation status (figure 4D).

Discussion

Using unbiased microarray analysis we report that IL-1β induces vascular bed specific transcriptional regulation in SMCs. Control (Normal) PASMCs display reduced inflammatory activation to IL-1β comparative to AoSMCs in vitro. mRNA and pathway analysis identified a dampened pro-inflammatory, pro-proliferative response to IL-1β in PASMCs compared to AoSMC which was lost upon loss of functional BMPR2. These findings are consistent with reports demonstrating beneficial effects of IL-1 receptor antagonist (IL-1ra) in animal models of PAH where BMPR2 is lowered such as the monocrotaline model. Furthermore, we demonstrate that BMPR2 signalling dysfunction results in increased inflammatory signalling and activation of mitogenic pathways such as PDGF which are inhibited in normal PASMC in-vitro but this does not occur in SMCs from the systemic vascular bed. This is an inherent part of the disease pathogenesis and our findings could go some way to explain why patients with BMPR2 mutations go on to develop pulmonary vascular remodelling without effects on their systemic vessels.

These findings are confirmed in the cells from lung transplant patients with and without disease causing BMPR2 mutations.
We report for the first time the additive effect for the loss of BMPR2 and administration of IL-1β on the catalogue of transcriptional activity. Mutant BMPR2 expression combined with IL-1β caused raised PVR associated with increased small pulmonary vessel muscularisation and correlated positively with serum IL-6 levels.

Therapeutic agents targeting IL-1β are in clinical use, of note anakinra, which has recently been trialled in therapeutic use in PAH. This gives reason for further investigation in to the role IL-1β may play in disease and late stage clinical trials using biologics (canukimumab) in cardiovascular disease gives further emphasis for insights into this potential therapeutic area.

Our data suggest that targeting IL-1β may be an effective therapeutic strategy for PAH treatment and that patient stratification according to IL-1β responsive signals could be useful for identifying patients with an increased likelihood of treatment response. An advantage of targeting IL-1β specifically is that this may mitigate against the risk of opportunistic infection by allowing other IL-1 to participate in host defence. Other recent research has shown that targeting inflammatory signalling in combination with increasing BMPR2 function is helpful in the treatment of PAH by the use in animal models of the drug FK506 (Tacrolimus) and a subsequent phase IIa clinical trial.

Manipulation of inflammatory cell infiltration and endothelial cell apoptosis by antagonizing the BLT1 receptor of leukotriene LTB₄ has also been shown to be effective in-vivo which backs up our findings that a combination of inflammation and BMPR2 dysfunction is important. Interestingly, the importance of neutrophil elastase (NE) in the pathobiology of PAH has been well recognized and recent work demonstrating NE mediated release of IL-1β may provide further evidence for a central role of IL-1β biology in the pathogenesis of PAH, and highlights additional evidence for the potential
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Figures

Figure 1
Figure 2

A

B

C

D

E

NT si  BMPr2 si

Mak si  BMPr2 si

Ntsi 236  288  537

I-SNE Dimensionality Reduction Outcome

-100

0

100

Fibroblast Reporter

% Increase in NF-kB

IL-1β

BMP4

Mock si

BMPr2 si
**Figure 3**

A. Heatmap showing gene expression levels under different conditions.

B. Bar charts illustrating the quantification of specific factors.

C. Comparison of gene expression levels across different groups.

D. Graphs depicting the relative quantity of specific proteins.

E. Comparison of gene expression levels with varying genetic types.

F. Bar charts showing the relative expression of different genes.

**Figure 4**

Diagram illustrating a timeline of dietary and pharmacological interventions:
- 0 weeks: Western diet with doxycycline
- 6 weeks: Western diet with doxycycline

Timeline:
- 12 x R899X: Placebo
- 12 x R899X: IL-1β (3ug/kg/day)
- 6 x WT: Placebo
- 6 x WT: IL-1β (3ug/kg/day)
Figure 5
Pathway analysis performed using signalling pathway impact analysis (SPIA) pathway analysis software on IL-1β stimulated in PASMC (a) AoSMCs (b) and PASMC in the presence of siRNA to BMPR2 (c) using SPIA code in R all gene information from arrays are taken into account and cross-referenced against known pathways. The Y-axis shows degree of perturbation against the X-axis plotting the number of differential expressed genes and each point on the graph is pathway expressed as a pathway number with decode table also shown.

Supplemental figure 2
Protein interactions in the IL-1β and BMPR2 pathways. BMP and IL-1β signalling pathways are linked through the co-localisation and co-expression of gene family members (http://www.genemania.com)