BTS/BLF/BALR Early career investigator symposium

T1 TNF MEDIATES ECTODOMAIN SHEDDING OF BMPR-II: A MECHANISM FOR INFLAMMATION AS A TRIGGER FOR PULMONARY ARTERIAL HYPERTENSION

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Background Mutations in BMP type II receptor (BMPR-II) account for 70% of heritable pulmonary arterial hypertension (PAH) cases, but low penetrance (~20%) in mutation carriers implies a 'second hit' is required for disease initiation. Inflammation has been implicated, yet the molecular mechanisms by which it influences pathology are unclear. Tumour necrosis factor alpha (TNF) has been reported to influence BMPR-II expression, yet the molecular interplay remains unclear.

Methods Human pulmonary arterial smooth muscle cells (PASMCs) were stimulated with TNF (1ng/mL). Biochemical (immunoprecipitation, western blotting), molecular biology (quantitative PCR, plasmid DNA transfection, site-directed mutagenesis, RNA interference) and pharmacological (metalloprotease, proteasome and lysosome inhibitors) approaches were used to assess the impact of TNF on BMPR-II and BMP signalling pathways. To assess this *in vivo*, two animal models of PAH were utilised: a mouse model overexpressing TNF specifically in the lung as well as a moncrotaline induced PAH (MCT-PAH) rat model.

Results TNF stimulation reduced BMPR-II mRNA and protein expression, leading to loss of BMP signalling, as evidenced by abrogated Smad 1/5 and ID1 activation. Notably, a low molecular weight form of BMPR-II accumulated in PASMC lysates following prolonged TNF exposure: identified as a C-terminal cleavage product of BMPR-II. Furthermore, the N-terminal ectodomain of BMPR-II could be immunoprecipitated from PASMC conditioned media and was quantified by ELISA. TNF increased expression of two A Disintegrin and Metalloproteinase Domain-containing proteins (ADAMs); ADAM10/17. Pharmacological blockade and RNA interference revealed both proteases were capable of BMPR-II cleavage. Mutation of the putative cleavage site restored BMP signalling. The cleaved ectodomain acted as a ligand trap, sequestering BMP ligands and inhibiting their signalling capacity. Proliferation assays revealed loss of BMP2/4 induced PASMC anti-proliferation in the presence of BMPR-II ectodomain. Finally, both animal models revealed reduced BMPR-II and c-terminal cleavage product in lung tissue - highlighting this event occurs *in vivo*.

Conclusions We identified a novel mechanism by which TNF impairs BMP signalling and promotes PASMC proliferation in the lung vasculature. TNFa may provide the critical link between inflammation and disease initiation in PAH. Our *in vivo* observations highlight TNF as a potential therapeutic target in PAH.

T2 VEGF SIGNALLING:DIFFERENCES IN ISOFORMS?

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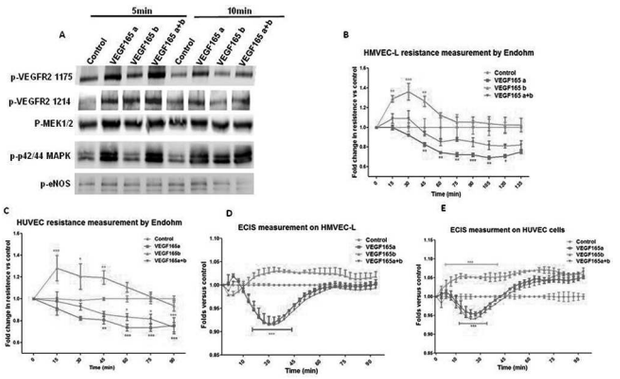
Vascular endothelial growth factor (VEGF) undergoes alternate splicing producing isoforms with differing functional



Abstract T2 Figure 1. HMVEC-L and HUVEC cells stimulated with 20 ng/ml of $VEGF_{165}$a, $VEGF_{165}$b, $VEGF_{165}$ a + b or without any stimulation (control) A, immunoblotting of primary HUMVEC-L treated for 5 to 10min and immunoblotted for the phosphorylation of p-VEGFR-2, p-MEK, p-MAPK and p-eNOS using phosphospecific antibodies (comparable results for HUVEC cells, data not shown). B C, Measurement of TEER by Endohm (inserts 0.4 m pore size). $VEGF_{165}$a reduces resistance (increased permeability) ***p < 0.001 (45min onwards) and $VEGF_{165}$b increases resistance (decrease permeability) **p < 0.01 (15 to 45min) compared with control. In combination $VEGF_{165}$b ameliorates $VEGF_{165}$a effect in HMVEC-L. D E, Electrical Cell-Substrate Impedance Sensor (ECIS) measurement (B well assay 8W10E +); $VEGF_{165}$a reduces the resistance (increased permeability) $VEGF_{165}$b increases resistance (decreases permeability) in comparison to control. Data were analysed using one-way ANOVA and Bonferroni post test analysis.

effects. The most biologically active and extensively studied isoform is $VEGF_{165}$. An isoform that causes inhibition of endothelial proliferation, migration and permeability, $VEGF_{165}$b has also been identified (Bates *et al*. 2002). We have previously investigated the downstream signalling mechanisms in response to $VEGF_{165}$ in pulmonary and systemic endothelial cells and have now compared these to the effects of $VEGF_{165}$b.

HUVEC and Human Lung Microvascular Endothelial Cells (HMVEC-L) were treated with both VEGF isoforms. Phosphorylation of VEGFR-2 (tyr^{1175} and tyr^{1214}) was measured along with phosphorylation/activation of pMEK1/2, p44/42MAPK (regulating cell proliferation) and eNOS (involved in cell permeability). We have previously shown the functional effects of $VEGF_{165}$/$VEGF_{165}$b on HMVEC-L proliferation (Varet *et al*. 2010). We have now explored the functional effects of $VEGF_{165}$/ $VEGF_{165}$b on cell permeability parameters by Endohm and Electrical Cell-Substrate Impedance Sensor (ECIS) measurements and modification in VE-cadherin cell distribution. The effects of the eNOS inhibitor L-NIO were also investigated.

$VEGF_{165}$ induced maximal phosphorylation of VEGFR-2 at tyr^{1175} and tyr^{1214} between 5 and 10min (>10 fold increase), $VEGF_{165}$b induced less than 5 fold increase compared to control. Comparable results for both isoforms were seen for activation of pMEK1/2, p42/44MAPK and eNOS. The two permeability assessments showed an increase in cells permeability due to $VEGF_{165}$ (HUVEC $p < 0.001$); (HMVEC-l $p < 0.01$) in contrast to $VEGF_{165}$b. This may be reflected by the differential changes in the cellular distribution of VE-cadherin induced in both cell types by VEGF isofroms. Treatment with L-NIO inhibited the effect of $VEGF_{165}$b suggesting a potential regulatory mechanism..

$VEGF_{165}$b induces differential responses to $VEGF_{165}$ in HUVEC and HMVEC-L. These observations suggest separate pathways for the regulation of mitogenesis and permeability which may be targeted.