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Increased Glutamine Metabolism is a Key Feature of the Metabolic Reprogramming in BMPR2 Mutant Pulmonary Endothelium

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Background & Hypothesis

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease of the pulmonary vasculature that ultimately results in right ventricular failure and death. The heritable form of PAH (HPAH) is attributable to loss of function mutations in the *bone morphogenic protein receptor type 2* (*BMPR2*) gene in the majority of cases, and many cases of idiopathic PAH are also related to impaired BMPR2 function. Though much is known about the genetics and epidemiology of PAH, the underlying molecular etiologies are complex and incompletely understood. A growing body of evidence suggests that abnormal cellular metabolism is a key contributing factor to the pathogenesis of PAH.

Several groups have demonstrated a shift in cellular metabolic program in PAH. Much of the published work has focused on a shift toward aerobic glycolysis in PAH, a phenomenon termed the "Warburg effect" in malignancy and thought to underlie increased cell proliferation and resistance to apoptosis. Our recent work has extended the metabolic characterization of BMPR2-mediated PAH and has shown significant shifts in the normal anaplerotic mechansims in BMPR2 mutant endothelium. In particular, we have shown increases in isocitrate dehydrogenase activity despite downstream depletion of Krebs cycle intermediates.

This led us to hypothesize that the flow of carbon to and through the Krebs cycle would be markedly altered in PAH, similar to what has been observed in several forms of malignancy. In particular, we hypothesized that glucose carbon flow into the Krebs cycle would be impaired, and that glutamine carbon flow into the Krebs cycle would be both increased and re-routed in BMPR2 mutant pulmonary endothelium compared to wild type pulmonary endothelium. Further, as this type of metabolic reprogramming has shown to be related to activation of hypoxia-inducible factor (HIF) in cancers and in other PAH models, we hypothesized that normoxic HIF activation would be associated with expression of a BMPR2 mutation.

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Methods

Cell Culture:
Pulmonary microvascular endothelial cells (PMVECs) were isolated from the lungs of wild-type and BMPR2 mutant mice ($BMPR2^{R899X}$, a mutation in the cytoplasmic tail of the receptor) crossed onto the Immortomouse background. Cells isolated from the Immortomouse express SV40 large T antigen when grown at 33°C in the presence of murine interferon-γ, allowing for prolonged maintenance in culture. When transitioned to 37°C in the absence of interferon-γ, cells revert to a primary culture phenotype. The BMPR2 mutant construct is an R899X mutation expressed under the control of the TetO7 promoter, with rtTA expressed under the control of the Rosa-26 promoter. Cells were transitioned to 37°C with doxycycline for at least 72 hours prior to assay, and all experiments were performed at ~80% confluence.

For a human PMVEC cell line, cells were stably transfected with either a native BMPR2 construct or a BMPR2 mutant construct expressing a BMPR2 mutant in the cytoplasmic tail of the receptor that was identified in a family with multiple members affected by HPAH and is analogous to that expressed in the murine PMVECs. Selection was maintained using G418 sulfate until > 12 hours before experiments.

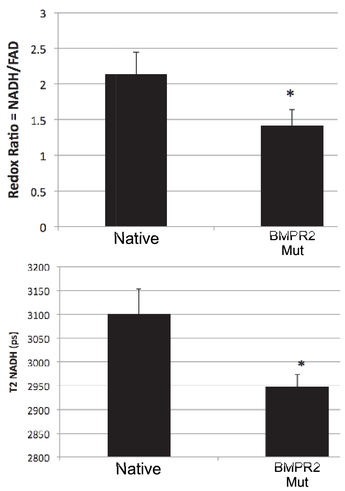
Determination of Optical Redox Ratio:
Wild-type and BMPR2 mutant PMVECs were grown to ~80% confluence in 35mm glass-bottom dishes. Using a custom-built two-photon imaging system in Vanderbilt's Biophotonics Laboratory, a titanium sapphire laser was used as the excitation source for NADH (750 nm) and FAD (890 nm) in live cells, with excitation and emission light coupled to an inverted scope with 40X water immersion objective. Emission spectra were collected, along with subsequent acquisition of fluorescence lifetime images using time-correlated single photon counting. After thresholding, the optical redox ratio was determined for each pixel in the fluorescence lifetime image, with an overall ratio calculated from the pixel average. Fluorescence decay was determined using SPCImage.

Extracellular Metabolite Fluxes:
Wild-type and BMPR2 mutant PMVECs were grown in culture and media samples collected at indicated timepoints. Glucose and lactate were quantified using a YSI 2300 Stat Plus Glucose and Lactate Analyzer, and glutamine were measured by HPLC and quantified by comparison to known internal standards.

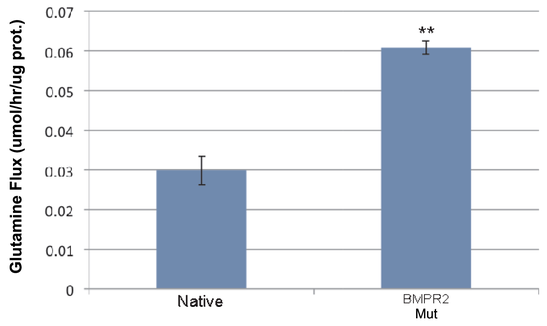
Stable Isotope Labelling Using $^{13}C_5$-L-Glutamine:
Wild-type and BMPR2 mutant PMVECs were grown in culture in standard endothelial cell growth medium. Media was then exchanged for a glutamine-free media supplemented with endothelial cell growth factors, 5% dialyzed FBS, and with fully labeled ^{13}C-L-glutamine added at a concentration of 1mM. After 24 hours, media was collected, and cells were harvested into ice-cold methanol with norvaline added as an internal standard. Following chemical derivatization, extracted metabolites were analyzed by mass spectrometry, with determination of stable isotope distributions used to confirm degree of label incorporation and parent-daughter ion spectra used to confirm identity of specific metabolites.

Cell Proliferation Assays:
Wild-type and BMPR2 mutant PMVECs were seeded at a density of 150,000 cells per well in glutamine-free media supplemented with 5% dialyzed FBS and with specific concentrations of L-glutamine added back to the media. Cells were maintained at 37°C and counted at the indicated timepoints using a Countess cell counter with live/dead determination using Trypan blue.

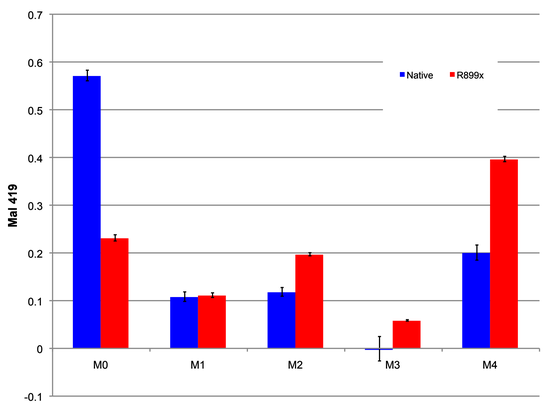
Results



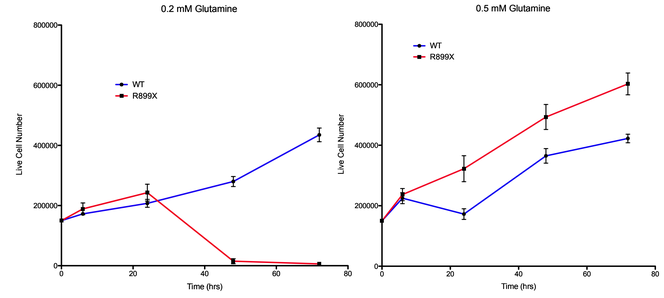
A human PMVEC cell line stably expressing either native BMPR2 (Native) or a cytoplasmic tail domain BMPR2 mutant (BMPR2 Mut) were analyzed by two-photon autofluorescence microscopy to quantify intracellular NADH and FAD. The ratio of these, termed the optical redox ratio, is an indicator of the relative activities of glycolysis/Krebs cycle and oxidative phosphorylation. BMPR2 mutant cells had a significantly lower optical redox ratio (n = 9, *p < 0.05 by rank sum test), consistent with less carbon flow through the Krebs cycle. Consistent with this, BMPR2 mutant cells also had a decreased fluorescence lifetime for NADH, indicating a different protein binding environment for NADH in the BMPR2 mutant PMVECs.



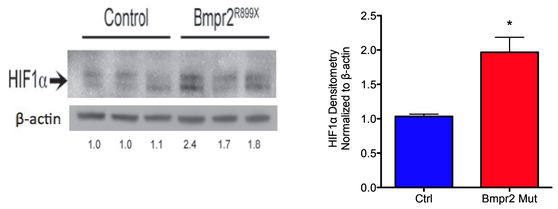
BMPR2 mutant PMVECs have a significantly higher glutamine uptake compared to PMVECs expressing native BMPR2. Media was collected and analyzed by HPLC at various timepoints over 24 hours to calculate an extracellular glutamine flux rate. The glutamine uptake rate for mutant BMPR2 endothelial cells was over twice that for wild-type cells (**p < 0.005 by Student's t-test). This was accompanied by a concomitant significant decrease in extracellular glucose uptake and in lactate production (data not shown), further supporting abnormal carbon flow through the Krebs cycle in BMPR2 mutants.



BMPR2 mutant (R899X) murine PMVECs not only take up more glutamine, but the flow of glutamine-derived carbon through the Krebs cycle is markedly different compared to wild-type (Native) PMVECs. Using $^{13}C_5$-L-glutamine, Krebs cycle intermediates were labeled for 24 hours, extracted, and analyzed by mass spectrometry as described under Methods. Shown are the representative relative amounts for the derivatized isotopomers of malate (*m/z* = 419 for parent ion). M0 indicates no label incorporation, with the ion coming entirely from non-glutamine carbon. M4 indicates full label incorporation, with all of the carbons present coming from glutamine. In wild-type PMVECs, nearly 60% of the Krebs cycle carbon is unlabeled, with only 20% full label incorporation. By contrast, in the R899X mutants, only 20-25% of the Krebs cycle carbon is unlabeled, with 40% being fully labeled. Moreover, there is evidence for "reverse" glutamine carbon flow in the mutant PMVECs (evidenced by the presence of the M3 ion).



Glutamine is required for efficient proliferation of BMPR2 mutant pulmonary endothelial cells. On the left, wild-type (WT, blue) and BMPR2 mutant (R899X, red) were plated at identical seeding densities in 0.2 mM total glutamine (typical cell culture conditions use 2 mM glutamine supplementation in addition to what is present in serum). Under glutamine restricted conditions, WT cells proliferate normally, whereas BMPR2 mutant PMVECs do not survive past 24 hours. By contrast, in 0.5 mM total glutamine, BMPR2 mutant PMVECs display enhanced proliferation compared to WT cells, which are unaffected by the increased glutamine availability.



Expression of mutant BMPR2 in PMVECs is associated with normoxic stabilization of HIF1α. Wild-type and BMPR2 mutant PMVECs were cultured under standard conditions, and lysates analyzed by Western blot for total HIF1α. Densitometry was normalized to beta-actin. Compared to control cells, BMPR2 mutant PMVECs had a significant increase in total HIF1α ($p < 0.05$) under normoxic conditions.

Conclusions & Future Directions

Expression of BMPR2 mutant isoforms in pulmonary endothelial cells results in a significant change in metabolic program. Glucose-derived carbon flux through the Krebs cycle decreases, and glutamine-derived carbon flux increases. The metabolic fate of glutamine carbon is also substantially changed in BMPR2 mutant endothelium. The increased glutamine is required for efficient proliferation of BMPR2 mutant cells, and this "glutamine addiction" is associated with normoxic stabilization of HIF1α, as has been seen in some malignancies. Thus, glutamine metabolism represents a relatively unexplored avenue for the development of novel diagnostics and therapeutics for PAH.