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A. Title of Research Project

PPAR-γ in DNA Damage Response in Pulmonary Arterial Hypertension

B. Background

Pulmonary arterial hypertension (PAH) is a severe disease characterized by occlusive muscularization of the pulmonary arterioles, and loss of distal arteries by endothelial cell (EC) apoptosis, features that impede blood flow in the lung (Rabinovitch, 2008). Expansion of smooth muscle cells (SMC) and dysregulated EC growth are characteristic of the most advanced pathology called plexygenic arteriopathy (Cool et al., 2005). Adults with PAH left untreated have a median survival of 2.8 years, and no treatment currently exists that can reverse the pathological changes associated with PAH making lung transplantation the sole curative treatment option (McLaughlin et al., 2004).

PPAR-γ and PAH

Bone morphongenetic protein receptor BMPR2 heterozygous mutations are found in nearly 70% of those with familial PAH, and 20% of those with idiopathic PAH (Aldred et al., 2010). Deletions of chromosome-8, and chromosome-13 have been found in PAECs from PAH patients, suggesting genomic instability (Masri et al., 2012). BMPR2 protein deficiency in cells has been linked to vulnerability to PAEC apoptosis and to the emergence of apoptosis resistant ECs that could transform into smooth muscle like cells, contributing to the plexogenic arteriopathy (Rabinovitch, 2008). Peroxisome proliferator activated receptor (PPAR)- γ is a transcription factor activated by BMPR2 receptor signaling whose activity facilitates the homeostasis of the pulmonary arterial vasculature. BMPs have been shown to activate PPAR-γ in ECs, allowing for the PPAR-γ : β-catenin complex to form, which promotes EC survival and in smooth muscle cells BMPR2 activated PPAR-γ to suppress abnormal growth (Alastalo et al., 2008).

PPAR-γ and MRN DNA Damage Response

By performing immunoprecipitation and mass spectrometry, Dr. Li of the Rabinovitch Lab, discovered that PPAR-γ interacts with the MRE-11/RAD-50/NBS1 (MRN) complex. The MRN complex functions as a DNA lesion sensor that assembles at DNA damage sites to recruit and activate the protein kinases ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) (Ciccia et al., 2010). ATM then activates γH2AX (to form p-γH2AX), a protein that forms a foci at the DNA damage site. The γH2AX foci recruits other proteins to induce DNA repair. ATM and ATR in turn phosphorylate (activate [p-]) other protein kinases, including CHK1 and CHK2 (to form pCHK1 and pCHK2) to facilitate DNA break repair. The downstream proteins p53 and CDC25 are also activated to halt the cell cycle and prevent replication of damaged DNA (Ciccia et al., 2010; Czornak, 2008).

C. Research Plan

Hypothesis

PPAR-γ is required for a normal DNA damage response in PAECs.

Specific Aim 1:

To establish an MRN mediated DNA damage response in 293T cells and PAECs.

Specific Aim 2:

To determine whether a PPAR-γ inhibitor prevents the activation of MRN mediated DNA damage response.

Methods and Procedures

Specific Aim 1(a)

Prior to experimentation with primary PAEC cells, which have limited proliferative potential, we optimized the experimental conditions in 293T cells (human embryonic kidney neuronal-like cells). 293T cells are a robust cell line that contain relatively large amounts of endogenous PPAR-γ, and are hyperproliferative. Hydroxyurea (HU), lipopolysaccharides (LPS), and doxorubicin (DoxR) will be each used to induce DNA damage evident at 6 hours. Protein will then be extracted using RIPA (radioimmunoprecipitation assay) buffer. Equal protein amounts will be analyzed by SDS PAGE and western blotting. Levels of p-γH2AX, pCHK1, pCHK2, total CHK1, and total CHK2 would be used to determine the DNA damage response.

Specific Aim 1(b)
Commercially available primary Lonza PAEC cells will then be treated with HU, LPS, and DoxR based on the conditions from aim 1(a). After extracting protein, and performing SDS PAGE and western blotting, levels of pCHK1, pCHK2, total CHK1, and total CHK2 will be used to determine the DNA damage response.

Specific Aim 2(a)
Based on the results of specific aim 1(a), treatment conditions will be chosen from HU, LPS, and DoxR based on the extent of DNA damage induced in the 293T cells. These cells will then be pre-treated or co-treated with GW9662, a synthetic agent capable of inhibiting PPAR-γ function (Seargent et al., 2004). After extracting protein, and performing SDS PAGE and western blotting, levels of p-γH2AX, pCHK1, pCHK2, total CHK1, and total CHK2 will be used to determine how the DNA damage response has been altered.

Specific Aim 2(b)
We will choose the agent that induces the greatest DNA damage response in 293T cells in specific aim 2(a) to promote DNA damage in the PAEC cells. PAEC cells will then be pre-treated or co-treated with GW9662. After extracting protein, and performing SDS PAGE and western blotting, levels of pCHK1, pCHK2, total CHK1, and total CHK2 will be used to determine how the DNA damage response has been altered.

Anticipated Results, Potential Pitfalls
We anticipated that with the addition of GW9662, cells would not activate CHK1 and CHK2 (pCHK1 and pCHK2). This would indicate a reduced DNA damage response as a result of the inhibited PPAR-γ.

Potential pitfalls of this experiment:

Drug Used: Drug response may differ between the 293T and the PAECs.
Testing other DNA damaging agents could provide a new treatment that induces sufficient DNA damage in both 293T and PAECs.

Treatment period: It is possible that the DNA damage response induced by the MRN complex is only delayed by PPAR-γ inhibition and this would not be evident at the 6 hour time-point. Reducing the time of treatment, or including multiple time points to the study may improve the opportunity to assess a delayed response.

Upstream effectors: Probing directly for ATM and ATR activity would provide a more accurate representation of MRN complex inhibition.

GW9662: GW9662, an inhibitor of PPAR-γ function may not affect PPAR-γ protein-protein interactions within the cell. Using small interfering RNA to knockdown PPAR-γ expression as an alternative to GW9662 could provide better results.

Significance
PPAR-γ, if demonstrated to interact with the MRN DNA damage complex, could potentially be used, with proper agonists, to rescue an impaired DNA damage response in PAH.

References

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