International Journal of Biochemistry and Cell Biology 92 (2017) 141–147

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Research paper

Hypoxia-induced activation of specific members of the NF-kB family and its relevance to pulmonary vascular remodeling

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ARTICLE INFO

Keywords:
Pulmonary hypertension
NF-kappa B
Vascular remodeling
Endothelial cells
Smooth muscle myocytes proliferation

ABSTRACT

Background and objective: Pulmonary Hypertension (pH) is a chronic progressive disease. Endothelial cells (EC) play a central and critical role in the initiation and progression of pH. The NF-κB family (NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and C-Rel) regulates a wide array of genes involved in inflammatory responses, cell proliferation, and survival. The involvement of specific NF-κB family members in the pathogenesis of hypoxia-induced pH remains to be determined.

The objective of this study was to assess the specific role of individual NF-κB family members in mediating endothelial cell responses to hypoxia and its downstream effect on smooth muscle cell proliferation.

Methods and results: NF-κB family members' expression were selectively reduced by siRNA in human pulmonary microvascular endothelial cells. Cells were then exposed to hypoxia (1%) for 24 h. Endothelin1, ICAM1 gene expression and Stat1 and Stat3 phosphorylation were assessed. Smooth muscle cells (SMC) proliferation was assessed by culturing them with EC conditioned media. Reduction of either NF-κB2 or RelA in EC, led to a significant decrease in Endothelin1 and ICAM1 gene expression. C-Rel knockdown resulted in a significant increase in phosphorylated STAT1; both C-Rel and RelA knockdown significantly decreased phosphorylated STAT3 in EC. There was a significant reduction in SMC proliferation, and AKT/ERK phosphorylation in SMC, when cultured in RelA knockdown, EC conditioned media.

Conclusion: RelA in EC plays crucial role in hypoxia induced vascular remodeling and development of pH. Targeting RelA in EC alleviates SMC proliferation as well as inflammation related processes.

1. Introduction

Pulmonary hypertension (PH) is a devastating disease with very poor prognosis. pH deteriorates rapidly after diagnosis, with an average survival time for primary pH of only 2.8 years, and an estimated 5-year survival of around 40%. Death rates associated with pH have continued to increase significantly during the past decade (George et al., 2014). pH can occur in association with chronic lung disorders, with hypoxia playing a pivotal role in the etiology, leading to pulmonary vessel constriction and even vessel narrowing by proliferation of endothelial cells (EC), smooth muscle cells (SMC) and adventitial fibroblasts. Each of these contribute to pulmonary vascular remodeling, which in turn chronically increase resistance to blood flow through the pulmonary circulation, leading to declining cardiac output and right ventricular failure (Stenmark et al., 2006; Tuder et al., 2007).

The mechanism of chronic hypoxia induced pulmonary vascular cell proliferation and remodeling is not fully understood (Stenmark et al., 2006; Tuder et al., 2007). Morphological changes to the intima are observed, but usually minimal (Reid and Meyrick, 1980). Hypoxia-induced remodeling is associated with medial hypertrophy, but direct stimulation of SMC proliferation by hypoxia remains controversial (Pak et al., 2007). While there are reports of hypoxia driven SMC

Abbreviations: pH, pulmonary hypertension; SMC, human pulmonary artery smooth muscle cells; EC, human pulmonary microvascular endothelial cells HPMECSt1.6R; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; RelA, v-rel avian reticuloendotheliosis viral oncogene homolog A; C-Rel, v-rel avian reticuloendotheliosis viral oncoggene homolog; Stat, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor; HIF1α, hypoxia-inducible factor alpha subunit; ICAM1, Intercellular Adhesion Molecule 1; ET1, endothelin-1

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http://dx.doi.org/10.1016/j.biocel.2017.09.022
Received 26 July 2017; Received in revised form 11 September 2017; Accepted 28 September 2017
Available online 04 October 2017
1357-2725/

proliferation (Frank et al., 2005; Preston et al., 2006), several *in vitro* studies have shown that hypoxia does not directly increase SMC proliferation (Dempsey et al., 1991; Lanner et al., 2005; Stiebellehner et al., 2003), or may actually decrease proliferation (Benitz et al., 1986; Rose et al., 2002).

Endothelium is known to play important roles in vascular remodeling through its intracellular NF-κB signaling (Saito et al., 2013). NF-κB is also a key regulator in vascular inflammatory processes, such as the induction of Intercellular Adhesion Molecule 1 (ICAM1) and other genes whose products mediate the interaction between the endothelium and circulating leukocytes (Khan et al., 1996; Kitamoto et al., 2000). In patients with idiopathic pH, there is an increase in NF-κB activation in macrophages and EC isolated from pulmonary arteries (Price et al., 2013). Hypoxia induces overexpression and release of Endothelin-1 (ET1) in EC. Once released, Endothelin-1 induces vascular cell proliferation (Chen and Oparil, 2000). STAT signaling pathways in EC are also involved with cell proliferation, survival and inflammatory processes associated with hypoxia and pH.

Sustained hypoxia enhanced NF-κB activity in a manner that was dependent upon the canonical signaling pathway (Oliver et al., 2009). In animals exposed to hypoxia, NF-κB levels was about 13-fold higher in the nuclear extract of the lungs of over control animals (Sarada et al., 2008). Once NF-κB is activated, it can mediate an inflammatory response caused by oxidative stress by inducing gene transcription of cytokines such as IL-1, IL-6, TNF-α, and adhesion molecules ICAM-1 (Reiterer et al., 2004). The biosynthesis and release of Endothelin-1 (ET-1) are regulated at the transcriptional level by NF-κB in mouse brain microvascular endothelial cells (Lin et al., 2013).

Recently, it was demonstrated that mice with endothelium-specific inhibition of functional nuclear factor-kB (NF-kB) (Brasier, 2010) are protected from atherosclerosis (Gareus et al., 2008), hypertension-induced renal damage (Henke et al., 2007), septic shock, and septic endothelial dysfunction (Ding et al., 2009). All these findings and many other findings, point towards endothelial NF-kB being as the key controller of the backyard fire in the vascular wall. NF-κB regulates the transcription of genes involved in inflammatory responses, cell growth control and apoptosis, which include cytokines, cell adhesion molecules, chemoattractant proteins, and growth factors (Ghosh and Karin, 2002). This precludes "blanket" inhibition of NF-κB as a clinical intervention. However, we believe that targeting the activity of a specific NF-κB protein will lead to a better understanding of the precise pathways involved in the pathogenesis of pH, and be the basis of a more defined and targeted therapeutic to reduce hypoxia induced pathology. In this study, our objective is to identify a specific NF-κB target in EC, which plays major role in hypoxia induced pH related key processes, such as ET-1 and ICAM1 expression and SMC proliferation.

2. Material and methods

2.1. Animals and hypoxia exposure

C57BL6 adult male mice were exposed to 10% hypoxia in a controlled hypoxia chamber (BioSpherix, Lacona, NY) for 21 days. Control mice were kept at room air. Animals were euthanized after 21 days and lungs were collected. Lungs were snap frozen in liquid nitrogen and kept at −80 °C until use. Animal protocol was reviewed and approved by IACUC committee of the Feinstein Institute for Medical Research.

2.2. Endothelial cell (ECs) culture and hypoxia exposure

The human pulmonary microvascular endothelial cell line (EC), HPMEC-ST1.6R was kindly provided by C. James Kirkpatrick, Institute of Pathology, Johannes-Gutenberg University, Germany. ECs were cultured using Endothelial Cell Growth Medium (Promocell, Heidelberg, Germany) under 5% CO_2 at 37 °C. Hypoxia was created by flushing the airtight hypoxia chamber (BioSpherix, Lacona, NY) with 95% Nitrogen and 5% CO_2 gas. For conditioned media to be used for SMC proliferation, media without growth factor was used, media was collected after 24 h hypoxia exposure, floating cells were removed using centrifugation and conditioned media was placed on SMC.

2.3. Transcription factor assay

Transcription factors were measured using TransAM® NFκB Family Kit (Active Motif, Carlsbad, CA). Active transcription factors were measured in total cell lysates and tissue lysates. The assay was carried out according to manufacturer's instruction. Protein assay was done using fluorescent ProStain™ Protein Quantification Kit (Active Motif, Carlsbad, CA).

2.4. siRNA transfection

EC were transfected with Silencer® Select Pre-Designed siRNAs for NFkB1, NFkB2, RELA, RELB, C-REL or Negative siRNA control using Lipofectamine RNAiMAX (Life Technologies, Grand Island, NY) without antibiotic for 4–6 h according to manufacturer's instruction. Transfection Medium was changed to full medium and cells were incubated another 48 h before hypoxia exposure.

2.5. RT-qPCR

Total RNA was extracted from EC using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Endothelin1 and ICAM1, mRNA expression was measured in ECs using LightCycler® 480 and LightCycler® 480 RNA Master Hydrolysis Probes kit (Roche Applied Science, Indianapolis, IN). Primers and probes were designed using Roche's Universal Probe Library Assay Design Center. Endothelin1 primers: Forward "tctctgctgtttgtggcttg" Reverse "gagctcagcgcctaagactg" UPL probe#50. ICAM1 primers: Forward "ccttcctcaccgtgtactgg" Reverse "agcgtagggtaaggttcttgc" UPL probe#71. HPRT1 was used as a Housekeeping gene.

2.6. Smooth muscle cell (SMC) culture

Primary human pulmonary artery smooth muscle cells (HPASMC) were purchased from Cell Application (San Diego, CA) and were cultured with SMC growth medium (Cell applications) under 5% CO_2 at 37 °C. For conditioned media experiments, SMC media was removed, cells were washed and SMC were incubated with hypoxia or room air exposed to EC conditioned media for 24 h at room air.

2.7. SMC cell proliferation assays

SMC proliferation was measured after 24 h incubation with EC conditioned media. Proliferation was measured using colorimetric Cell Proliferation ELISA BrdU kit (Roche Applied Science, Indianapolis, IN). SMC were incubated with BrdU together with EC conditioned media for 24 h. After 24 h media was removed cells were fixed using FixDenat reagent and assay was performed as per the manufacturer's instructions. SMC proliferation was also assessed by cell count. SMCs were trypsinized, washed with PBS and counted using hemocytometer and trypan blue exclusion method.

2.8. Western blot

p52, p50, p65, RelB, C-Rel, p-stat1, Stat1, p-stat3, Stat3, Akt, pAkt, Erk, pErk and secondary antibodies were purchased from Santa Cruz (Santa Curz Biotechnoloies, Santa cruz, CA, USA). Proteins were extracted using RIPA buffer supplemented with protease and phosphatase inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Proteins were resolved on SDS-PAGE and blotted on the PVDF membrane. Beta actin (Genscript, Piscataway, NJ) was used as a loading control.