ONLINE SUPPLEMENT

Inhibition of NF-κB in the lungs prevents monocrotaline-induced pulmonary hypertension in mice

Li Li¹, Chuanyu Wei¹, Il-Kwon Kim³, Yvonne Janssen-Heininger² and Sudhiranjan Gupta*

Division of Molecular Cardiology, Department of Medicine, College of Medicine, Texas A & M Health Science Center; Scott & White; Central Texas Veterans Health Care System, Temple, Texas, USA, ²University of Vermont, Burlington VT, USA

Running title: NF-κB modulates pulmonary hypertension in mice

¹ Equally contributed to the work
³ Current address: Innovative cell and gene therapy center, International St. Mary’s Hospital, Incheon, South Korea

*Corresponding author.

Tel: 1-254-743-2465

Fax: 1-254-743-0165

Email address: sgupta@medicine.tamhsc.edu
Supplemental Material and Methods
Generation of Transgenic Mice Overexpressing the IκBα Mutant Gene (IKBM)

Generation of the CC-10 promoter driven IκBα mutant mice has been previously described. Age- and sex-matched wild-type (WT) mice of C57BL/6 background served as controls. Twelve-week-old male mice (~25 g) were used for experiments. The studies were conducted with the approval of Institutional Animal Care and Use Committee at the Texas A&M Health Science Center and Scott &White Hospital.

Induction of Pulmonary Arterial Hypertension (PAH)

The MCT-induced PAH mouse model was developed as described previously with modifications. Briefly, WT and IKBM transgenic mice received an intraperitoneal injection of MCT (80 mg/kg body wt) every 20 days for 3 times. Vehicle (normal saline)-injected WT and IKBM transgenic mice served as controls. A total of six mice were studied in each group. All mice were fed standard rodent chow and provided water ad libitum. The animals were studied on the twentieth day of the last MCT administration.

Determination of RV Pressure and RVH

The RV pressure was determined using iWorks System, pressure catheter (CATH-SCI-1200) as described previously.

Morphological Examination

Lungs and RVs were fixed in 10% phosphate-buffered formalin, stained with hematoxylin and eosin (H&E) and Masson’s trichrome, respectively; as described previously. Ventricular histological sections were stained with wheat germ agglutinin (WGA) conjugated to tetramethylrhodamineisothiocyanate to determine the myocyte cross-sectional areas. Images of RV cardiomyocytes were captured by the Leica TCS SP5 confocal system (Leica, Mannheim, Germany) and analyzed using the Image J software from the National Institutes of Health.

Culture of Lung Microvascular Endothelial Cells (ECs)

Lung microvascular ECs were purchased from VEC Technologies (Rensselaer, NY, USA). Cells were cultured and passaged in MCDB-131 complete medium (VEC Technology, NY). Lung ECs were serum-free for 24 h before stimulation with TGF-β1.

Small Interfering RNA (siRNA) and Plasmid Transfection

ECs were cultured to 80% confluence and transfected with siRNA of interest by use of MISSION® siRNA Transfection Reagent (Sigma-Aldrich Co., St. Louis, MO, USA). The siRNA targeting BMPR2 and scrambled siRNA were
from Sigma-Aldrich. Plasmid transfection was performed as described previously.3

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)
RNA was extracted from the lung and RV tissue of WT, IKBM, WT+MCT, and IKBM+MCT mice using an RNEasy kit (Qiagen, Valencia, CA, USA), following the manufacturer’s instructions. The qRT-PCR was performed using gene-specific primers as described previously.2

Western Blot Analysis
The lung and RV heart tissue were pulverized in liquid nitrogen; the cytosolic and nuclear proteins were extracted using nuclear and cytosolic extraction reagents (NE-PER, Pierce, Rockford, IL). Western blotting and the subsequent quantification of each blots was performed, as described previously.2 The primary antibodies used in this study include BMPR2, Notch-3, Id1, Id3, histone (all from Santa Cruz Biotechnologies, Santa Cruz, CA); NF-κB p65 subunit, Bax, Bcl2, cleaved caspase-3, and GAPDH (all from Cell Signaling Technologies, Danvers, MA).

Immunofluorescence Staining
Immunohistofluorescence was performed as previously described.2 Sections or ECs were incubated with anti-NF-κB p65 antibody (Cell Signaling) or stained with the antibodies anti-CD31 (Abcam, Cambridge, MA) and anti-α-SMA (Sigma, St. Louis, MO) overnight at 4°C, then secondary antibody for 2 h at 37°C. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Fluorescence images were captured by use of the Leica TCS SP5 confocal system. All sections were examined and at least three to five images from each section were acquired.

Statistical Analysis
All experiments were performed at least three times for each determination. Data are expressed as the means ± SE and were analyzed using one-way ANOVA and secondary analysis or significance with Newman-Keuls Multiple comparison test, using Prism 5.0 GraphPad software (GraphPad, San Diego, CA). P<0.05 was considered statistically significant.
References


**Supplemental Results**

Figure S1 IκBα protein expression in the cytoplasmic and nuclear fraction of lung tissue from WT and IKBM mice. A, Representative Western blots showing the levels of IκBα in the cytoplasmic fraction of lung tissue. GAPDH served as internal loading controls for the cytoplasmic fractions. B, Normalized band intensity quantification showing fold change of IκBα in the cytoplasmic fraction of lung tissue. C, Representative Western blots showing the levels of IκBα in the nuclear fraction of lung tissue. Histone 4 served as internal loading controls for the nuclear fractions. D, Normalized band intensity quantification showing fold change of IκBα in the nuclear fraction of lung tissue. Data are expressed as the means± SE from 4 independent mice. **P <0.01
compared with the WT mice.

Figure S2 TGF-β1 induces EndMT in lung microvascular ECs. Lung ECs were incubated with 10 ng/ml TGF-β1 for 1, 3, 6, and 9 days. A, Representative fluorescent microscopy images of α-SMA staining in lung ECs. Red fluorescence represents α-SMA, and blue fluorescence is DAPI nuclei staining. B, Representative Western blots showing protein expression of CD31 and α-SMA in lung ECs. GAPDH served as internal loading control. C and D, Normalized band intensity quantification showing the levels of CD31 and α-SMA. Data are expressed as means ± SE from 3 independent experiments. *P < 0.05 and **P < 0.01 compared with the 0 d control.