Inhibition of Nuclear Factor-κB in the Lungs Prevents Monocrotaline-Induced Pulmonary Hypertension in Mice

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Abstract—Pulmonary arterial hypertension (PAH) is a devastating cardiopulmonary disorder with significant morbidity and mortality in patients with various lung and heart diseases. PAH is characterized by vascular obstruction which leads to a sustained increased pulmonary vascular resistance, vascular remodeling, and right ventricular hypertrophy and failure. Limited PAH therapies indicate that novel approaches are urgently needed for the treatment of PAH. Nuclear factor-κB (NF-κB) has been shown to play an important role in different cardiac pathologies; however, the role of NF-κB remains limited in the setting of PAH. Here, we investigated whether NF-κB inhibition in the lungs using Club (Clara) cell-10 promoter driving IκBα mutant had any effect in monocrotaline (MCT)-induced PAH mouse model. Our data revealed that MCT-induced PAH and right ventricular hypertrophy were associated with NF-κB activation, inflammatory response, and altered expression of bone morphogenetic protein receptor 2, inhibitor of differentiation, and Notch-3 signaling molecules in wild-type mice; and all these alterations were prevented in IκBα mutant mice treated with MCT. Moreover, endothelial cell apoptosis and endothelial-to-mesenchymal transition occurred in the lungs of MCT-treated wild-type mice and were restored in IκBα mutant+MCT mice, indicating an association with NF-κB signaling. In lung microvascular endothelial cells, IκBα (AA) mutant plasmid restored the decreased bone morphogenetic protein receptor 2 protein level and reversed the endothelial-to-mesenchymal transition process induced by transforming growth factor-β1. We conclude that NF-κB regulates bone morphogenetic protein receptor 2–inhibitor of differentiation–Notch-3 axis genes and the subsequent endothelial cell apoptosis and endothelial-to-mesenchymal transition events in the lungs, providing new mechanistic information about MCT-induced PAH and right ventricular hypertrophy. (Hypertension. 2014;63:00-00.) • Online Data Supplement

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Pulmonary arterial hypertension (PAH) is characterized by multicellular vascular lesions which obstruct and obliterate pulmonary arteries. The occluded vessels impede blood flow and increase right ventricular (RV) afterload leading to RV hypertrophy (RVH) and RV failure.1,2 The pathogenesis of PAH is multifactorial and involves vascular cell remodeling including pulmonary arterial endothelial cell (EC) dysfunction and pulmonary arterial smooth muscle cell proliferation.3,4 Treatment regimens for PAH include inhaled nitric oxide, endothelin receptor antagonists, which fail to fully reverse this disease.5 Identifying key pathways is, therefore, required for the development of new targeted therapeutics.

Nuclear factor-κB (NF-κB) is a key transcriptional regulator in various cardiac disorders (myocardial infarction, hypertrophy, and dilated cardiomyopathy, etc.),6,7 but the role of NF-κB remains limited in PAH-induced vascular remodeling and RVH. Recent few reports advocate the critical role of NF-κB in the progression or setting of PAH,8,9 but the underlying mechanism of monocrotaline (MCT)-induced PAH and its progression to the development of RVH remains elusive. MCT is a plant alkaloid contained in the seeds of Croton tiglium and its metabolite, MCT pyrrole, injures the ECs of pulmonary blood vessels.10 It is suggested that MCT treatment causes direct damage of ECs, increases alveolar capillary permeability, and induces inflammation that triggers the development of PAH and further progresses to RVH.11–13 Despite its frequent use for many decades, the basic mechanism underlying PAH induction by MCT has not been fully understood.

We demonstrated previously that MCT-treated PAH-induced RVH was attenuated in cardiac-specific IκBα triple mutant transgenic mice, compared with wild-type (WT) mice, suggesting a protective role of NF-κB in RVH.14 We identified bone morphogenetic protein (BMP), BMP receptor (BMPR), inhibitor of differentiation (Id), SMAD, and the Notch (BMP–SMAD–Id–Notch) axis as an integral signaling
mechanism required for PAH-induced RVH. The enhanced NF-κB activation in MCT-induced PAH appears to promote expression of inflammatory mediators, and interventions that inhibit NF-κB activation have been speculated to rescue the phenomena. Because transgenic manipulation in mouse is a powerful genetic approach to analyze the mechanism of diseases, we, therefore, use this approach to examine the role of NF-κB in MCT-induced PAH and RVH. This study is designed to inhibit NF-κB activation in the lungs using Club (Clara) cell-10 (CC-10) promoter driving IxBα mutant (IKBM) or super-repressor gene.

Although this transgene is under transcriptional control of the CC-10 promoter which primarily inhibits NF-κB activation in airway epithelial cells, it has been further evident to the existence of biochemically distinct populations of Club (Clara) cells within the rodent lung. Therefore, we anticipate the inhibition of NF-κB activation in the lungs.

Here, we investigated the effect of inhibition of NF-κB in the lungs of IKBM on MCT-induced PAH and RVH. We chose MCT-induced rodent model because MCT triggers inflammation and ECs damage which lead to vascular remodeling and development of PAH within 2 months. Our data show for the first time that inhibition of NF-κB in IKBM protects the mice from MCT-induced PAH and RVH, compared with the WT mice.

Materials and Methods
Detailed experimental procedures and statistical analysis for the present study are described in the online-only Data Supplement.

Results
MCT-Induced PAH Is Alleviated in IKBM Mice
CC-10 promoter–driven IKBM mice were used to investigate the functional role of NF-κB in MCT-induced PAH. IKBM mice showed robust protein expression of IxBα in the cytoplasmic fraction of lung tissues, compared with WT mice (Figure S1A and S1B in the online-only Data Supplement). No significant difference of IxBα protein expression in the nuclear fraction of lung tissues was observed between WT and IKBM mice (Figure S1C and S1D).

The pathogenesis of MCT-induced PAH was evaluated through RV pressure measurement. There was a significant increase in the RV pressure in the MCT-treated WT mice, compared with the untreated WT mice (36.6±0.69 versus 27.8±0.24 mm Hg; P<0.01). Compared with the MCT-treated WT mice, the MCT-treated IKBM mice showed significant reduction in RV pressure (31.3±0.76 versus 36.6±0.69 mm Hg; P<0.05; Figure 1A).

Western blot analysis of nuclear proteins showed the increased NF-κB p65 level in lungs of MCT-treated WT mice, compared with the untreated WT mice (2.65-fold; P<0.01). MCT-treated IKBM mice showed significant reduction of nuclear NF-κB p65 protein level, compared with the MCT-treated WT mice (1.25-fold; P<0.05; Figure 1B and 1C). Immunofluorescence analysis revealed translocation of NF-κB p65 protein into the nucleus in MCT-treated WT mice, which was prevented significantly in the MCT-treated IKBM mice (Figure 1D). Together, our data indicate that MCT treatment activates NF-κB in the lungs, which may trigger the proinflammatory response for the progression of PAH and is prevented in IKBM mice.

MCT-Induced Lung Injury and Expression of Proinflammatory Cytokine Genes Are Prevented in IKBM Mice
The histological alterations in MCT-induced lung damage in WT and IKBM mice are demonstrated in Figure 2A and 2B. MCT injection triggered intense infiltration of macrophages in WT mice. The normal structure of alveoli was lost in many areas. These maladaptive changes were alleviated in the MCT-treated IKBM mice (Figure 2A). Masson trichrome staining showed that there was obvious collagen deposition in the pulmonary interstitium in the MCT-treated WT mice. The MCT-induced lung interstitial fibrosis was significantly alleviated in the MCT-treated IKBM mice (Figure 2B and 2C). There was no significant morphological alteration in the lung tissue of IKBM mice, compared with the untreated WT mice (Figure 2A and 2B).

The mRNA expression of the proinflammatory cytokine/chemokine genes, interleukin (IL)-6, tumor necrosis factor-α, IL-1β, and regulated on activation normal T-cell expressed and presumably secreted was increased to 5.49- (P<0.01), 5.62- (P<0.01), 5.90- (P<0.01), and 6.50-fold (P<0.01), respectively, in lungs of the MCT-treated WT mice, compared with
Figure 2. Monocrotaline (MCT)-induced lung injury and expression of proinflammatory cytokine genes was prevented in IκB-κα mutant (IKBM) mice. A. Representative hematoxylin and eosin staining images showing MCT-induced lung injury in wild-type (WT) and IKBM mice. B. Representative Masson trichrome staining showing the MCT-induced collagen deposition in WT and IKBM mice. C. Histogram of connective tissue percentage revealed interstitial fibrosis in the MCT-treated WT and IKBM mice. Blue staining indicates connective tissue (n=6; 6 fields in each sample were scanned and averaged). D. The mRNA level of proinflammatory cytokine genes [interleukin (IL)-6, tumor necrosis factor-α (TNF-α), IL-1β], and regulated on activation normal T cell expressed and presumably secreted (RANTES) in MCT-exposed WT and IKBM mice was determined by quantitative reverse transcription polymerase chain reaction. Data are expressed as means±SE from 3 independent mice. **P<0.01 compared with the WT mice. #P<0.05 and ##P<0.01 compared with the WT+MCT mice.

MCT Treatment Alters the Expression of BMPR2, Smad8, Id Family, and Notch-3 Genes in the Lungs

To determine the alteration of cell signaling molecules contributed to the MCT-induced PAH in WT and IKBM mice, the expression of BMP–Smad–Id–Notch signaling axis molecules was determined in the lung tissue. The mRNA expression of BMP2, BMPR2, and Smad8 genes was reduced by 59.92% (P<0.05), 54.56% (P<0.05), and 57.03% (P<0.05), respectively, in the MCT-treated WT mice, compared with the untreated WT mice. The expression of BMPR2 and Smad8 was restored in the MCT-treated IKBM mice, compared with the MCT-treated WT mice (P<0.05). The mRNA expression of BMP2 gene was not restored in the MCT-treated IKBM mice, indicating that inhibition of NF-kB did not affect expression of the ligand BMP2 (Figure 3A). Also, the mRNA expression of Id1 and Id3 genes was reduced by 65.50% (P<0.05) and 57.04% (P<0.05), respectively, in the MCT-treated WT mice, compared with the untreated WT mice. The down-regulation of both Id genes was restored in the MCT-treated IKBM mice (P<0.05; Figure 3B). MCT treatment increased the mRNA expression of Notch-3 gene to 2.44-fold in the WT mice (P<0.05), compared with untreated WT counterpart. The MCT-treated IKBM mice showed 87.70% downregulation of the Notch-3 gene, compared with the MCT-treated WT mice (P<0.05; Figure 3B).

We also observed a significant downregulation in protein expression of BMPR2, Id1, and Id3 by 52.52% (P<0.01), 53.32% (P<0.01), and 54.31% (P<0.01), respectively, in the MCT-treated WT mice, and a marked upregulation of Notch-3 to 2.33-fold (P<0.01). The MCT-treated IKBM mice showed restored protein expression of these molecules, compared with the MCT-treated WT mice (BMPR2, Id3, and Notch-3, P<0.05; Id1, P<0.01; Figure 3C and 3D).

MCT Treatment Alters the Expression of Apoptotic Family Genes in Lung

Expression of antiapoptotic gene Bcl2 was downregulated by 56.26% in the MCT-treated WT mice, compared with the untreated WT mice (P<0.05). The expression of Bcl2 was increased to 2.33-fold in the MCT-treated IKBM mice, compared with the MCT-treated WT mice (P<0.05), indicating the restoration of Bcl2 mRNA expression. Expression of the proapoptotic genes, such as caspase-3 and Bax, was increased to 2.28- (P<0.05) and 2.29-fold (P<0.05), respectively, in the MCT-treated WT mice, compared with the untreated WT mice. The expression of Bax and caspase-3 was reduced in the MCT-treated IKBM mice by 75.37% (P<0.05).
and 76.53% (P<0.05), respectively, compared with the MCT-treated WT mice (Figure 4A). Representative Western blot showed upregulation of Bax, downregulation of Bcl2, and increased level of cleaved caspase-3 in the MCT-treated WT mice (Figure 4B). The Bax/Bcl2 ratio (indicator of apoptosis) and cleaved caspase-3 were increased to 3.32- (P<0.01) and 3.02-fold (P<0.01), respectively, in the MCT-treated WT mice, compared with the untreated WT mice, but was reduced significantly in the MCT-treated IKBM mice (Bax/Bcl2 ratio, P<0.01; cleaved caspase-3, P<0.05; Figure 4C).

Apoptosis of ECs was determined by double immunofluorescence staining for CD31 and terminal uridine nick end labeling (Figure 4D). In the WT and IKBM mice, the median percentage of apoptotic ECs was 1.36% and 1.03%, respectively. MCT treatment in the WT mice resulted in increased percentage of apoptotic ECs to 18.39% (P<0.01). The MCT-treated IKBM mice showed a significant reduction of apoptotic ECs, compared with the MCT-treated WT mice (P<0.01; Figure 4E).

MCT-Induced Endothelial-to-Mesenchymal Transition Is Prevented in Lung of IKBM Mice

Quantitative reverse transcription polymerase chain reaction exhibited a marked reduction in the EC markers, CD31 and vascular endothelial cadherin, by 58.82% (P<0.05) and 63.09% (P<0.05), respectively, in the MCT-treated WT mice. This reduction was restored in the MCT-treated IKBM mice (P<0.05). We also observed a 5.12-fold increase in the

Figure 4. Monocrotaline (MCT) treatment alters the expression of apoptotic family genes in lung. A, The mRNA level of Bcl2, Bax, and caspase-3 in MCT-exposed wild-type (WT) and IκBα mutant (IKBM) mice was determined by quantitative reverse transcription polymerase chain reaction. Data are expressed as means±SE from 3 independent mice. B, Representative Western blots showing protein expression of Bax, Bcl2, and cleaved caspase-3 in MCT-exposed WT and IKBM mice. C, Normalized band intensity quantification showing Bax/Bcl2 ratio and the level of cleaved caspase-3. Data are expressed as means±SE from 4 independent mice. D, Representative fluorescent microscopy images of CD31 and terminal uridine nick end labeling co-staining in the lung tissue of WT, WT+MCT, IKBM, and IKBM+MCT mice. Green fluorescence represents apoptotic nuclei, blue fluorescence is 4,6-diamidino-2-phenylindole nuclei staining, and red fluorescence represents CD31. E, Bar graphs of terminal uridine nick end labeling-positive endothelial cells in the lung tissue of WT, WT+MCT, IKBM, and IKBM+MCT mice. Data are expressed as means±SE from 4 independent mice. *P<0.05 and **P<0.01 compared with the WT mice. #P<0.05 and ##P<0.01 compared with the WT+MCT mice.

Figure 5. Monocrotaline (MCT)-induced endothelial-to-mesenchymal transition is prevented in lung of IκBα mutant (IKBM) mice. A, The mRNA level of CD31, vascular endothelial (VE) cadherin, and α-smooth muscle actin (α-SMA) in MCT-exposed wild-type (WT) and IKBM mice was determined by quantitative reverse transcription polymerase chain reaction. Data are expressed as means±SE from 3 independent mice. B, Representative fluorescent microscopy images of CD31 and α-SMA co-staining in the lung tissue of WT, WT+MCT, IKBM, and IKBM+MCT mice. Green fluorescence represents α-SMA, blue fluorescence is 4,6-diamidino-2-phenylindole nuclei staining, and red fluorescence represents CD31. C, Averaged bar graphs of CD31 and α-SMA density in the lung tissue of WT, WT+MCT, IKBM, and IKBM+MCT mice (n=6; 6 fields in each sample were scanned and averaged). *P<0.05 and **P<0.01 compared with the WT mice. #P<0.05 and ##P<0.01 compared with the WT+MCT mice.
expression of α-smooth muscle actin (α-SMA) in the MCT-treated WT mice, compared with the untreated WT mice (P<0.01). This upregulation was also observed to a lower degree in the MCT-treated IKBM mice (P<0.01; Figure 5A).

To determine whether MCT treatment induces endothelial-to-mesenchymal transition (EndMT) in pulmonary ECs, we used double immunofluorescence staining for CD31 and α-SMA. Red-stained CD31-positive ECs were detected in lung of the untreated WT and IKBM mice, whereas the green-stained α-SMA-positive ECs appeared in small numbers. MCT-treated WT mice presented weak expression of CD31 but gained strong expression of α-SMA, indicating EndMT was induced in the process of MCT-triggered PAH. We observed restored expression of CD31 and decreased expression of α-SMA in the MCT-treated IKBM mice, compared with the MCT-treated WT mice (Figure 5B and 5C).

NF-κB Inhibition Reverses Transforming Growth Factor-β1-Induced EndMT in Lung Microvascular ECs

Increased mesenchymal cells with spindle shape and enhanced α-SMA staining were observed when lung ECs were incubated with 10 ng/mL transforming growth factor-β1 (TGF-β1) for 6 and 9 days (Figure S2A). Protein expression of CD31 was reduced to 54.93% (P<0.05), 37.98% (P<0.01), and 25.02% (P<0.01), respectively, at 3, 6, and 9 days (Figure S2B and S2C). We also observed significant increase of α-SMA protein expression to 1.73-, 2.28- (P<0.01), and 2.85-fold (P<0.01), respectively, at 3, 6, and 9 days (Figure S2B and S2D). TGF-β1–induced EndMT characterized by spindle shape, increased α-SMA staining, and decreased CD31 staining was reversed when lung ECs were transfected with IκBα (AA) mutant plasmid (Figure 6A). Western blot analysis showed that IκBα (AA) mutant plasmid reversed the reduced CD31 expression and the increased α-SMA expression in response to TGF-β1 (Figure 6B and 6C).

NF-κB Inhibition Restores TGF-β1–Reduced BMPR2 Expression in Lung Microvascular ECs

It has been reported that stimulation of BMPR2 signaling partially ameliorates the process of EndMT even in the ongoing presence of TGF-β1.23 In the present study, lung ECs were transfected with scrambled or BMPR2 small interfering RNA to determine the role of BMPR2 in TGF-β1–induced EndMT process. BMPR2 protein expression was reduced by 61.84% when transfected with 25 nmol/L BMPR2 small interfering RNA for 72 hours, compared with ECs transfected with scrambled small interfering RNA (P<0.01; Figure 7A). Treatment with BMPR2 small interfering RNA facilitated the TGF-β1–induced EndMT process, demonstrated by further increased α-SMA and decreased CD31 expression (Figure 7B, 7C, and 7D). We also found that the mRNA and protein expression of BMPR2 was reduced by 37.82% (P<0.05) and 46.02% (P<0.05), respectively, when treated with TGF-β1 for 1 day. Incubation with TGF-β1 for 3, 6, and 9 days induced further decrease of BMPR2 expression (Figure 7E and 7F). Transfection of IκBα (AA) mutant plasmid restored the decreased BMPR2 protein level induced by TGF-β1 (Figure 7G).

MCT-Induced RVH Is Alleviated in IKBM Mice

RVH was determined by a ratio of RV weight to left ventricle (LV) plus inter ventricular septum (S) weight (RV/LV+S). There was a significant increase in the RV/LV+S ratio in the MCT-treated WT mouse (0.39±0.05; P<0.01), compared with the untreated WT (0.20±0.02) or IKBM (0.23±0.02) mice. The MCT-treated IKBM mice showed an RV/LV+S ratio of 0.27±0.01, which was significantly reduced compared with the MCT-treated WT mice (P<0.01; Figure 8A).

Hematoxylin and eosin staining showed hypertrophied cardiomyocytes in the RV of the MCT-treated WT mice compared with the untreated WT group, and this maladaptive change was restored in the MCT-treated IKBM mice (Figure 8B). The representative images from the wheat germ agglutinin staining of hearts from WT, WT+MCT, IKBM, and IKBM+MCT depicting the effect of MCT treatment on cardiomyocytes cross-sectional area are shown in Figure 8C. Wheat germ agglutinin staining showed a 1.75-fold increase in cardiomyocytes cross-sectional area in the MCT-treated WT mice (P<0.01). The MCT-induced RV cardiomyocytes hypertrophy was significantly alleviated in the MCT-treated IKBM mice (P<0.01; Figure 8D). Gene expression of atrial natriuretic peptide, brain natriuretic peptide, and β-myosin heavy chain was increased to 3.44- (P<0.01), 3.28- (P<0.01), and 3.00-fold (P<0.01), respectively, in the MCT-treated
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WT mice, compared with the untreated WT mice. The MCT-treated IKBM mice showed only 1.60- (P<0.01), 1.64-(P<0.05), and 1.39-fold (P<0.05) increase in the mRNA expression of atrial natriuretic peptide, brain natriuretic peptide, and β-myosin heavy chain, respectively, which was significantly reduced when compared with the MCT-treated WT mice (Figure 8E).

MCT Treatment Alters Expression of BMPR2, Id1, Id3, and Notch-3 in Heart

Western blot analysis of nuclear proteins showed a 2.02-fold increase of NF-κB p65 subunit level in the RV of the MCT-exposed wild-type (WT) mice, compared with the untreated WT mice (P<0.01). The MCT-treated IKBM mice showed only 1.07-fold increase in NF-κB p65 subunit, which was significantly

Figure 7. Nuclear factor-κB inhibition restores transforming growth factor-β1 (TGF-β1)-reduced bone morphogenetic protein receptor 2 (BMPR2) expression in lung microvascular endothelial cells (ECs). A, Lung ECs were transfected with BMPR2 or scrambled small interfering RNA (siRNA) (25 nmol/L) for 48 hours. Representative Western blots and averaged bar graphs showing protein expression of BMPR2. GAPDH served as an internal control. B to D, Lung ECs were transfected with 25 nmol/L BMPR2 or scrambled siRNA for 48 hours and then incubated with 10 ng/mL TGF-β1 for 9 days. B, Representative fluorescent microscopy images of CD31 and α-smooth muscle actin (α-SMA) staining in lung ECs. Red fluorescence represents CD31 and blue fluorescence is 4,6-diamidino-2-phenylindole nuclei staining. C, Representative Western blots showing protein expression of CD31 and α-SMA in lung ECs. D, Normalized band intensity quantification showing level of CD31 and α-SMA proteins. Data are expressed as means±SE from 3 independent experiments. **P<0.01 compared with cells transfected with scrambled siRNA only. #P<0.05 compared with the TGF-β1-treated cells. E and F, Lung ECs were treated with 10 ng/mL TGF-β1 for 1, 3, 6, and 9 days. E, The mRNA level of BMPR2 was determined by quantitative reverse transcription polymerase chain reaction. F, Representative Western blots and averaged bar graphs showing protein expression of BMPR2, GAPDH served as an internal control. Data are expressed as means±SE from 3 independent experiments. *P<0.05 and **P<0.01 compared with the 0 days control. G, Lung ECs were transfected with 500 ng vehicle or IκBα (AA) mutant plasmid for 48 hours and then incubated with 10 ng/mL TGF-β1 for 3 days. Representative Western blots and averaged bar graphs showing protein expression of BMPR2, GAPDH served as an internal control. Data are expressed as means±SE from 3 independent experiments. **P<0.01 compared with cells transfected with vehicle only. ##P<0.01 compared with the TGF-β1-treated cells.

Figure 8. Monocrotaline (MCT)-induced right ventricular hypertrophy (RVH) is alleviated in IκBα mutant (IKBM) mice. A, The ratio of RV weight to left ventricle (LV) plus inter ventricular septum (S) weight (RV/(LV+S)) (index of RVH) of MCT-exposed wild-type (WT) and IKBM mice. Data are expressed as means±SE from 6 independent mice. B, Representative hematoxylin and eosin staining showing the MCT-induced morphological change in the RV from WT and IKBM mice. C, Representative WGA staining for RV transverse section of WT, WT+MCT, IKBM, and IKBM+MCT mice. D, Quantification of cardiomyocytes cross-sectional area derived from wheat germ agglutinin staining (n=4). E, The mRNA level of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC) in MCT-exposed WT and IKBM mice was determined by quantitative reverse transcription polymerase chain reaction. *P<0.05 compared with the WT mice. #P<0.05 and ##P<0.01 compared with the WT+MCT mice.
lower than the MCT-treated WT mice (*P<0.01; Figure 9A and 9B). MCT treatment in the WT mice resulted in dysregulated expression of BMPR2, Id1, Id3, and Notch-3 genes. The mRNA expression of BMPR2, Id1, and Id3 was reduced by 57.34% (*P<0.01), 79.11% (**P<0.01), and 76.82% (***P<0.01), respectively, in the RV of the MCT-treated WT mice, compared with the untreated WT mice. The reduction of these genes was restored in the MCT-treated IKBM mice (P<0.05). The expression of Notch-3 was increased to 3.11-fold (**P<0.01) in the MCT-treated WT mice and was restored in the MCT-treated IKBM mice (P<0.05; Figure 9C). Western blot analysis showed downregulation of BMPR2 (P<0.01), Id1 (P<0.05), and Id3 (P<0.01) and upregulation of Notch-3 (P<0.01) in the MCT-treated WT mice, which was markedly reversed in the MCT-treated IKBM mice (BMPR2, Id3, and Notch-3, P<0.01; Id1, P<0.05; Figure 9D and 9E).

**Discussion**

Our results demonstrate for the first time that inactivation of NF-κB in the lungs prevents MCT-induced PAH and RVH. The protective mechanism seems to be associated with restoration of BMPR2–Id–Notch-3 axis molecules, attenuation of inflammatory response, reduction of cell death in lung ECs, and reversal of EndMT process. Inhibition of NF-κB by pharmacological intervention or genetic manipulation or by nanoparticle (decoy of NF-κB) delivery has been reported to show protection, but this report provides for the first time that genetic inhibition of NF-κB in the lungs prevents the progression of PAH and RVH. Our study provides the following evidences in regards to the mechanism of MCT-induced PAH and RVH.

We showed that injection of MCT triggered PAH as evidenced by increased RV pressure in WT mice. MCT treatment is known to damage the pulmonary ECs which are critical for induction of PAH. It is suggested that MCT-induced endothelial damage elicits dramatic accumulation of infiltrating molecules which then triggers development and progression of PAH. Our data corroborated with these findings that we observed a significant infiltration of macrophages and accumulation of inflammatory molecules (tumor necrosis factor-α, IL-6, IL-1β), and regulated on activation normal T-cell expressed and presumably secreted) in the WT mice treated with MCT. These maladaptive changes were reversed in MCT+IKBM group, indicating a protective role of NF-κB in the lungs. Our data further provide a new evidence of accumulation of collagen as determined by Masson trichrome staining in the pulmonary interstitium of MCT-treated WT mice, which was significantly reduced in the MCT+IKBM group. Importantly, MCT treatment activated NF-κB in the lungs of WT mice and was significantly inhibited in the MCT+IKBM group. Together, our data indicate that inhibition of NF-κB in the lungs reduces the endothelial damage, attenuates the expression of infiltrating molecules, and restores the RV pressure.

Our study further confirmed the critical role of BMPR2–Id–Notch-3 axis genes in MCT-induced PAH. Previously, we demonstrated that cardiac-specific inhibition of NF-κB attenuated MCT-induced PAH and RVH by regulating BMPR2–Id–Notch-3 axis gene. Here, we show a direct association of NF-κB with BMPR2–Id–Notch-3 axis in the lungs of MCT-induced PAH. It is evident from the literature that BMPR2 signaling plays a critical role in the pathogenesis of PAH and familial PAH as well. Reduced BMPR2 expression occurs in MCT-induced and chronic hypoxic-induced rat models of PAH, and delivery of BMPR2 by gene therapy attenuates the disease in both models. Our data corroborated with these data. We observed a significant loss of BMPR2 in MCT-treated WT mice, which was restored in IKBM+MCT group. In addition, we observed a new association between NF-κB and Id protein which is now considered to be a novel player in PAH. Id proteins (Id1–Id4) are major downstream transcriptional targets of BMP signaling. We and others have previously identified the Id family of transcription factors as important functional targets of BMP signaling, with relevance to PAH. In this report, we further confirmed that both Id1 and Id3 were downregulated in the lungs along with BMPR2 in WT+MCT group and were restored in MCT+IKBM group. We also demonstrated that Notch-3, which is critical in PAH, was significantly reduced in MCT+IKBM group, compared with the WT+MCT group. Collectively, our data provide a strong evidence for the association of BMPR2–Id–Notch-3...
axis with MCT-induced PAH. Although this association is efficiently regulated by NF-xB, the mechanistic link between BMPR2–Id–Notch-3 axis and NF-xB during development of pulmonary vascular disease remains unclear and warrants future investigation. Taken together, our findings provide a rationale to consider the development of therapies based on BMPR2–Id–Notch-3 axis modulation.

The EC apoptosis has been shown to be associated with the pathophysiology of PAH and may trigger smooth muscle cell growth. Our data presenting the occurrence of apoptosis in ECs in the WT+MCT group support few earlier reports that endothelial apoptosis plays a role in the development of PAH. But, the role of NF-xB in this setting is currently unknown. In this regard, the reduction in the percentage of apoptotic ECs in MCT+IKBM group provides an alternative mechanism for clarifying the role of NF-xB in PAH. Growing evidence indicates that BMPR2 treatment reduces apoptosis under serum starvation and supports the notion that loss-of-function mutations of BMPR2 enhance pulmonary EC apoptosis that may initiate a possible mechanism in the pathogenesis of PAH. Our data showing the apoptotic event in the ECs may correlate with the reduction of BMPR2 function, which destroys the integrity of the endothelial barrier and contributes to endothelial dysfunction.

EndMT is a complex biological process in which ECs lose their surface marker protein, such as CD31 and vascular endothelial cadherin, and acquire mesenchymal phenotype, such as α-SMA. EndMT has also been investigated for its potential role in vascular remodeling and the fibrotic lung disease. In our study, we also observed a loss of vascular endothelial cadherin and CD31 and a gain of α-SMA expression in MCT-induced PAH, which was reversed in MCT+IKBM group. This is the first report that distinctly showed that NF-xB regulated this critical phenotype switch in the pathogenesis of PAH. A recent study shows that TGF-β1–induced EndMT in human pulmonary microvascular ECs is associated with reduced BMPR2 expression. EndMT is partially ameliorated by stimulating BMPR2 signaling even in the presence of TGF-β1. To corroborate our in vivo findings with in vitro analysis, we tested the effect of depletion of BMPR2 in mouse lung ECs stimulated with TGF-β1. Our data showed that BMPR2 depletion induced further remarkable increase in α-SMA level and marked attenuation of CD31 in the presence of TGF-β1, indicating a key role of BMPR2 in EndMT process. To further reveal the role of NF-xB in BMPR2 regulation, our data provide the evidence for the first time that BMPR2 was regulated by NF-xB under the similar setting of in vitro analysis. These results together may allow us to formulate a testable hypothesis for the pathogenesis of PAH in humans. A dysregulation of BMPR2 signaling may initiate pulmonary EC apoptosis and EndMT and further accelerate the flow and shear stress in the remaining vessels and exaggerate the effect.

Finally, we provide the evidence that MCT-induced PAH, which culminated into the development of RVH, was significantly attenuated in MCT+IKBM group. Our study showed that MCT treatment triggered the development of RVH in WT mice and was evidenced by an increase in cardiomyocytes cross-sectional area. The morphological changes were associated with enhanced activation of NF-xB, alteration of BMPR2–Id–Notch-3 axis genes, and upregulation of hypertrophy marker genes such as atrial natriuretic peptide, brain natriuretic peptide, and β-myosin heavy chain. We observed a significant reduction of NF-xB activation in the RV, attenuation of cardiomyocytes cross-sectional area, restoration of BMPR2–Id–Notch-3 axis genes, and significant reduction of hypertrophy marker genes in the MCT+IKBM mice, suggesting a direct link between NF-xB and PAH-induced RVH.

Our study reflects a functional significance of NF-xB inhibition in the lungs of MCT-induced PAH and RVH rodent model. Our data reveal the involvement of inflammatory response and BMPR2–Id–Notch-3 axis signaling in this process, which may trigger apoptotic and EndMT events in the lungs. Having determined the involvement of these signaling pathways, future studies are still necessary to examine whether the changes presented here reflect the outcome of MCT stimulation and not the cause of RVH, which may be a limitation. Prevention of above remodeling by NF-xB inhibition in the lungs may indicate a positive association between lung and heart in the pathogenesis of PAH.

In conclusion, our study identifies the importance of NF-xB in regulating BMPR2–Id–Notch-3 axis genes, EC apoptosis, and EndMT in the lungs, providing new mechanistic information about MCT-induced PAH and RVH. Our data suggest that NF-xB contributes a primary role in the pathogenesis of PAH and, thus, offers a target for therapeutic intervention in PAH and RVH.

Perspective

PAH results in RVH, a progressive RV failure, and low cardiac output leading to increase in morbidity and mortality because therapies are limited. Our study showed that inhibition of NF-xB in the lungs prevented RVH in MCT-induced PAH mouse model. Furthermore, a novel role of NF-xB in modulating EC death, EndMT, and BMPR2 signaling provides new mechanistic input and may offer novel therapeutic intervention to treat the deadly disease. We can speculate that limiting EC death and reversing the EndMT process during the pathological process of PAH may be used for at least part of the beneficial effects to treat the PAH and RVH.

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Disclosures

None

References


**What Is New?**

- This is the first report to demonstrate that inactivation of nuclear factor-κB in the lungs prevents right ventricular hypertrophy in monocrotaline-induced pulmonary arterial hypertension (PAH) mouse model targeting bone morphogenetic protein receptor 2–inhibitor of differentiation–Notch-3 and EndMT.

**What Is Relevant?**

- Bone morphogenetic protein receptor 2–inhibitor of differentiation–Notch-3 axis and endothelial-to-mesenchymal transition is the novel target for PAH.

**Summary**

Our study identifies the importance of nuclear factor-κB in regulating bone morphogenetic protein receptor 2–inhibitor of differentiation–Notch-3 axis genes, endothelial cell apoptosis, and endothelial-to-mesenchymal transition in the lungs, providing new mechanistic information about monocrotaline-induced PAH and right ventricular hypertrophy. Our data suggest that nuclear factor-κB contributes a primary role in the pathogenesis of PAH and, thus, offers a target for therapeutic intervention in PAH and right ventricular hypertrophy.
Inhibition of Nuclear Factor-κB in the Lungs Prevents Monocrotaline-Induced Pulmonary Hypertension in Mice

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

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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. 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.

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, Bcl\(_2\), 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


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.