

## Abstract

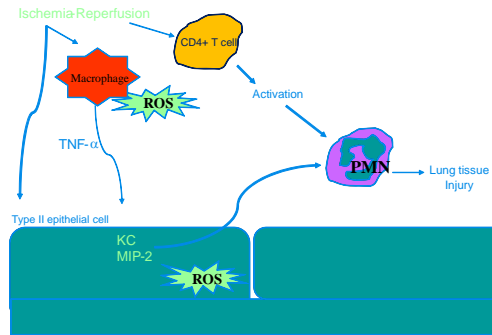
**Introduction:** Lung IR injury involves a complex interaction of various lung cell types, such as CD4+ T cells, macrophages and type II epithelial cells, via an oxidative stress-dependent mechanism. This study investigates the role of NADPH oxidase-dependent oxidative stress mechanisms in the initiation of lung IR injury.

**Methods:** An *in vivo* mouse left lung IR injury model was employed. Ncf1 mutant mice (which fail to produce superoxide) and C57BL/6 mice underwent either sham left thoracotomy or 1h left hilar occlusion followed by 2h reperfusion. At the end of reperfusion, an isolated, buffer-perfused lung system was used to evaluate pulmonary function. Lung injury was evaluated by measuring vascular permeability (Evans blue dye method) and lung edema (lung wet/dry ratio). An *in vitro* model of hypoxia-reoxygenation was also utilized in which RAW264.7 macrophages and MLE12 cells were exposed to 1 h hypoxia (5% O<sub>2</sub>) followed by 1 h reoxygenation. Cells were then loaded with dichlorofluorescein (DCF) dye to measure ROS generation. The antioxidant enzymatic activity was measured by evaluating catalase activity after H/R.

**Results:** After I/R, Ncf1 KO mice were markedly protected as compared to wild-type (WT) mice as indicated by significantly reduced lung injury (decreased vascular permeability and lung edema) and improved lung function (increased pulmonary compliance and decreased airway resistance and pulmonary artery pressure). WT mice treated with Apocynin (an NADPH oxidase inhibitor) also displayed significant protection in lung injury and function.

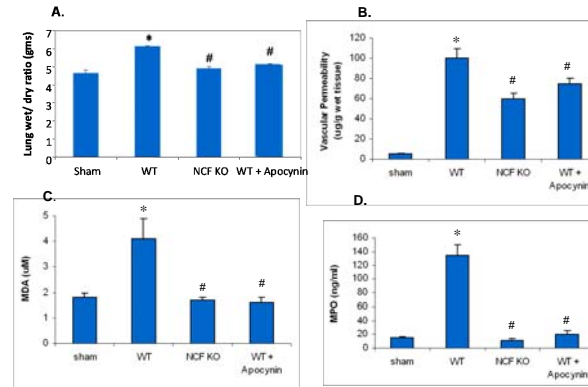
**Conclusion:** NADPH oxidase plays an important role in the progression of lung IR injury via an oxidative stress-dependent mechanism. The correlation of pro-inflammatory and oxidative stress-dependent signaling mechanisms in lung cells such as alveolar macrophages and type II epithelial cells, is likely to be a key event in the tissue injury after lung I/R.

## Results



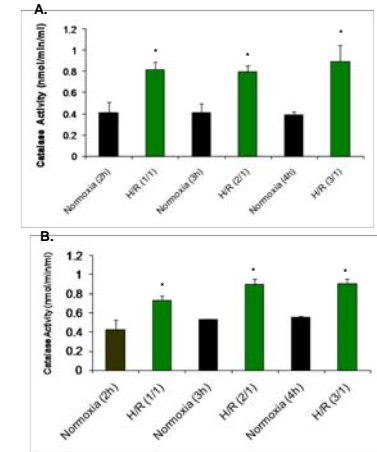
**Figure 1.** Hypothetical model depicting ischemia-reperfusion-induced pro-inflammatory and oxidative stress-dependent pathways following lung ischemia-reperfusion injury. PMN, neutrophils; ROS, reactive oxygen species.

## Results



**Figure 3.** Lung I/R injury is attenuated by NADPH oxidase inhibition. Lung edema (A) and vascular permeability (B) are markedly increased after lung I/R in WT mice. Ncf KO mice and WT mice treated with Apocynin display significantly decreased lung edema and vascular permeability after lung I/R. Malondialdehyde (MDA) (C) and myeloperoxidase (MPO) (D) activity in BAL fluid was markedly increased in WT mice after lung I/R and was significantly attenuated in Ncf KO mice and WT mice treated with Apocynin. \*P<0.05 WT I/R vs. sham; #P<0.01 NCF KO I/R and WT+Apocynin vs. WT I/R.

## Results



**Figure 5.** Catalase Activity Assay. The antioxidant catalase enzymatic activity was measured to determine oxidative stress generation in MLE12 and RAW264.7 cells after H/R. H/R induced a multi-fold activation of catalase in MLE12 (A) and RAW264.7 cells (B), at different time periods. \*p<0.05 vs. respective controls.

## Materials & Methods

**In vivo lung IRI.** C57BL/6 or Ncf1 KO mice underwent left lung ischemia by occlusion of the left hilum for 1 h after left thoracotomy. Five minutes before reperfusion animals were re-intubated and the suture occlusion was removed. Mice were extubated, allowed to awaken, and returned to their cage for 2 h. After 2 h reperfusion the animals were re-anesthetized and pulmonary function was measured. Sham animals received thoracotomy without hilar occlusion.

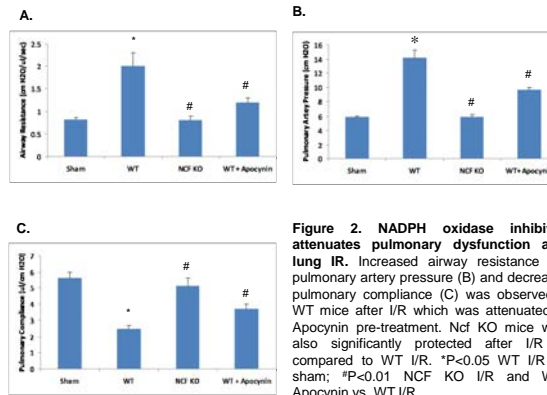
**Isolated, buffer-perfused mouse lung system:** Pulmonary function was evaluated using an isolated, buffer-perfused mouse lung system at the end of scheduled reperfusion. Isolated lungs were perfused with Krebs-Henseleit buffer for 10 min. Hemodynamic and pulmonary function parameters were recorded throughout this period by the PULMODYN data acquisition system (Hugo Sachs Elektronik).

**Bronchoalveolar (BAL) fluid collection:** After perfusion, the lungs were lavaged with 0.5 ml saline for three times and the BAL fluid was collected and centrifuged (1500 g for 15 min at 4°C).

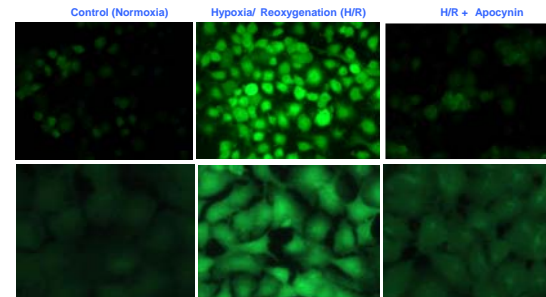
**Lung wet-to-dry ratio:** Lung wet-to-dry ratio was used as an indicator of pulmonary edema wherein the lower lobe of the right lung from each animal was harvested, weighed and placed in a vacuum oven (at 58°C) until a stable, dry weight was achieved. The ratio of lung wet weight to dry weight was then calculated.

**Vascular permeability assay:** Evans blue extravasation technique was used to determine vascular permeability. Absorption of Evans blue was measured at 620 nm in supernatants from homogenized lung tissues.

**Myeloperoxidase (MPO) measurement:** MPO was measured in BAL fluid as an indicator of neutrophil infiltration into alveolar spaces. The quantitative measurement of MPO was calculated by the mean absorbance values of each sample at 450 nm, and the final concentrations are expressed as ng/ml.



**Figure 2.** NADPH oxidase inhibition attenuates pulmonary dysfunction after lung IR. Increased airway resistance (A), pulmonary artery pressure (B) and decreased pulmonary compliance (C) was observed in WT mice after I/R which was attenuated by Apocynin pre-treatment. Ncf KO mice were also significantly protected after I/R as compared to WT I/R. \*P<0.05 WT I/R vs. sham; #P<0.01 NCF KO I/R and WT+Apocynin vs. WT I/R.



**Figure 4.** ROS generation in MLE12 and RAW 264.7 cells. MLE12 cells (top row) and RAW264.7 cells (bottom row) exposed to hypoxia/reoxygenation for 1h/1h displayed a marked increase in ROS generation which was significantly attenuated by Apocynin (NADPH oxidase inhibitor) (600μM) pretreatment.

## Summary

- Lung I/R injury is mediated by oxidative stress-dependent mechanisms involving NADPH oxidase.
- NADPH oxidase inhibition protects lung function and injury after I/R and attenuates I/R-mediated ROS generation.
- The correlation of pro-inflammatory and oxidative stress-dependent mechanisms in various lung cell type such as CD4+ T lymphocytes, alveolar macrophages and type II epithelial cells is likely a pivotal factor in the initiation and progression of pulmonary I/R injury.