Alveolar epithelial cell activation by hypoxia-reoxygenation entails oxidative stress-dependent mechanisms

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Abstract

- Pulmonary ischemia-reperfusion injury entails a complex interaction of molecular signaling mechanisms including pro-inflammatory cytokines (TNF-α, MIP-2 and KC), oxidative stress, and redox-sensitive transcription factors (NF-κB and AP-1).
- This study focuses on elucidating these signaling pathways in alveolar epithelial cells and their interactions with alveolar macrophages via TNF-α.
- Increased reactive oxygen species (ROS) production was observed in MLE12 alveolar epithelial cells and RAW264.7 macrophages after exposure to hypoxia/reoxygenation (H/R) compared to normoxic controls. In addition, MLE12 cells exposed to TNF-α also showed a significant induction of ROS as compared to controls.
- The TNF-α-induced cell death (38.9 ± 2.4% after 18hr and 60 ± 2.6% after 24hr) may be mediated via caspase-3 activation.
- H/R also induces a multi-fold activation of calpain and catalase, as compared to normoxic controls, in MLE12 and RAW264.7 cells.
- The pro-inflammatory cytokine/chemokine expression by alveolar epithelial cells after hypoxia-reoxygenation, and concurrent TNF-α-induced cell death, may be mediated by an oxidative stress-dependent mechanism.

Methods

Cell culture: RAW264.7 and MLE12 cells were grown in DMEM (high glucose) and DMEM F12 containing 10% FBS and 1% penicillin/streptomycin, respectively. Cultures were placed in a humidified, sealed hypoxic chamber (Billups-Rothenberg Inc., Del Mar, CA) which was then purged with 95% N_2 and 5% CO_2 for 25 min to establish hypoxia. The chamber was placed in a cell culture incubator for 1h, and reoxygenation was achieved by removing the plates from the hypoxic chamber and placing them in a normoxic, humidified incubator for 1h. The partial percentage of O_2 in the culture media after hypoxia was 5% compared to a normoxic pO_2 of 21%. Pretreatment of cells with anti-TNF-α neutralizing antibody (1mg/ml) was done 1h prior to hypoxic exposure.
ROS analysis: Intracellular ROS production was measured by using the dichlorofluorescein diacetate dye (DCFH/DA) (Molecular Probes). After exposure to H/R (1h/1h) or TNF-a (1h), cells were loaded with the DCF dye (10 mM) for 15 min at room temperature and the intracellular ROS generation was assessed by confocal microscopy.
MTT assay: Cells were washed twice with cold PBS and incubated in media containing MTT dye (Sigma; 0.5 mg/mL) for 2 hr. After aspiration of the medium, the crystals were dissolved with 0.1 N HCl in isopropyl alcohol and absorbance was measured at 570 nm (background wavelength 630 nm). Results are presented as percentage of survival, using control as 100%.
Caspase activity: Caspase-3 activity was measured by using the fluoregenic enzyme substrates, z-DEVD-AFC as instructed in the caspase-3 activity assay kit (Molecular Probes). The samples were read in a fluorometer equipped with a 400 nm excitation filter and 505 nm emission filter. Pretreatment with N-acetylcysteine (1 mM) was done 1h prior to TNF-α treatment.
Calpain activity: The fluorometric assay for calpain activity was used to quantify the changes in calpain activity as per the manufacturers protocol (BioVision Inc.). The detection of cleavage of calpain substrate, Ac-LLY-AFC, was quantified using a fluorometer with a 400 nm excitation filter and 505 nm emission filter.
Catalase activity: The catalase enzymatic activity assay was performed as per the manufacturers protocol (Calbiochem). The enzymatic activity was determined using a fluorometer at an absorbance of 540 nm.

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Hypothesis

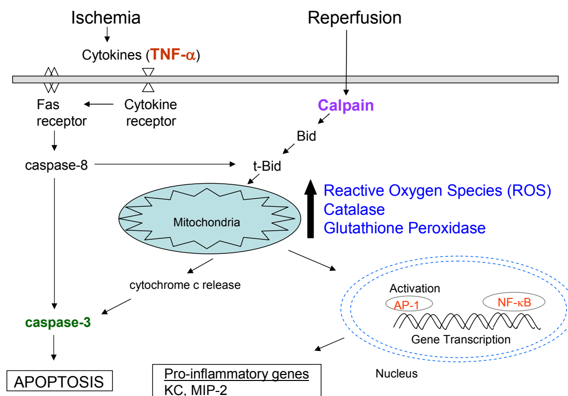


Fig. 1. Hypothetical model depicting ischemia-reperfusion-induced pro-inflammatory and oxidative stress-dependent pathways in alveolar type II epithelial cells following lung ischemia-reperfusion injury.

Results

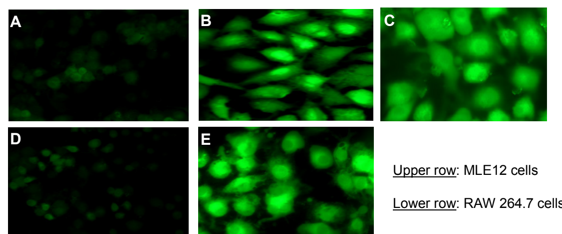


Fig. 2. ROS generation in MLE12 and RAW 264.7 cells. MLE12 cells exposed to H/R (1h/1h) (B) or TNF-α (20 ng/ml for 1h) (C) showed a significant induction of ROS as compared to normoxic control (A). H/R-exposed RAW264.7 cells (E) also showed a significant ROS generation as compared to the normoxic control (D).

Results

Figure 2. Calpain Activity. H/R induced a multi-fold activation of calpain in MLE12 and RAW264.7 cells, at different time periods. Also, TNF-α treatment (4 h) of MLE12 cells resulted in significant calpain activation as compared to controls. *p<0.05 vs. control.

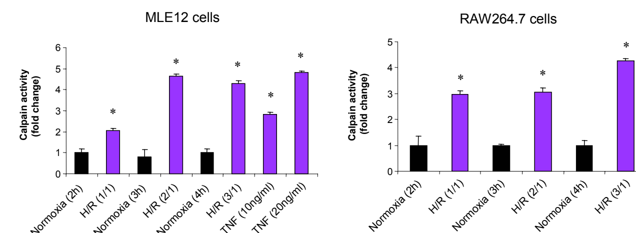
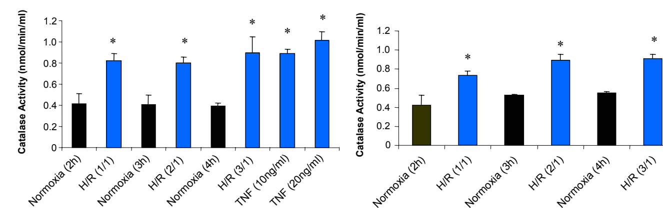


Figure 3. Catalase Activity. H/R induced a multi-fold activation of catalase in MLE12 and RAW264.7 cells at different time periods. Also, TNF-α treatment (4 h) of MLE12 cells resulted in significant catalase activation as compared to controls. *p<0.05 vs. control.



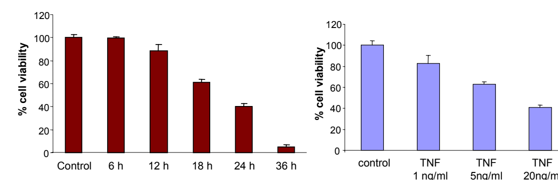


Figure 4. MTT Assay. Cell viability was measured after TNF-α treatment of MLE12 cells. TNF-α-induced cell death of MLE12 cells which was time dependent (**left**) and dose dependent (**right**).

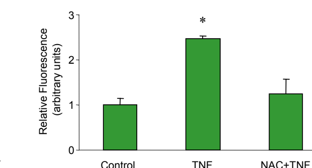


Figure 5. Caspase-3 Activity. TNF-α (20 ng/ml for 12 h) induced caspase-3 activation in MLE12 cells which is significantly ameliorated by pretreatment with N-acetylcysteine (NAC). *p<0.05 vs. control.

Summary

- Hypoxia-reoxygenation induces ROS generation in alveolar type II epithelial cells and alveolar macrophages.
- TNF-α (likely from alveolar macrophages) also contributes to the oxidative stress in type II epithelial cells.
- Activation of cysteine proteases like calpain and involvement of oxidative stress may be an important mechanism in the genesis of pro-inflammatory cytokines/chemokines in lung ischemia-reperfusion injury.