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Oxidative stress induced necroptosis activation is involved in the pathogenesis of hyperoxic acute lung injury

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ABSTRACT

Necroptosis has been found to be involved in the pathogenesis of some lung diseases, but its role in hyperoxic acute lung injury (HALI) is still unclear. This study aimed to investigate contribution of necroptosis to the pathogenesis of HALI induced by hyperbaric hyperoxia exposure in a rat model. Rats were divided into control group, HALI group, Nec-1 (necroptosis inhibitor) group and edaravone group. Rats were exposed to pure oxygen at 250 kPa for 6 h to induce HALI. At 30 min before hyperoxia exposure, rats were intraperitoneally injected with Nec-1 or edaravone, and sacrificed at 24 h after hyperoxia exposure. Lung injury was evaluated by histology, lung water to dry ratio (W/D) and bronchoalveolar lavage fluid (BALF) biochemistry; the serum and plasma oxidative stress, expression of RIP1, RIP3 and MLKL, and interaction between RIP1 and RIP3 were determined. Results showed hyperoxia exposure significantly caused damage to lung and increased necroptotic cells and the expression of RIP1, RIP3 and MLKL. Edaravone pre-treatment not only inhibited the oxidative stress in HALI, but also reduced necroptotic cells, decreased the expression of RIP1, RIP3 and MLKL and improved lung pathology. Nec-1 pretreatment inhibited necroptosis and improved lung pathology, but had little influence on oxidative stress. This study suggests hyperoxia exposure induces oxidative stress may activate necroptosis, involving in the pathology of HALI, and strategies targeting necroptosis may become promising treatments for HALI.

1. Introduction

Oxygen is indispensable for human life. Clinically, oxygen therapy has been one of the main supportive modalities in case of hypoxemia. However, excess oxygen is harmful for human body [1]. Oxygen has affinity for electrons and thus has the potential to cause damage to cells and tissues [2]. It has been confirmed that exposure to oxygen at a partial pressure higher than normal atmosphere for enough time may compromise the defense of human body, causing oxygen toxicity [3], and the pulmonary changes caused by oxygen toxicity are also known as the Lorrain Smith effect. Generally, exposure to oxygen at a partial pressure higher than 50 kPa for a long time may cause hyperoxic acute lung injury (HALI), which is pathologically characterized by the diffuse alveolar epithelial damage, vascular endothelial cell swelling, increased pulmonary microvascular permeability, and infiltration of a large number of neutrophils into the lung [4]. Although there is no epidemiological study about HALI, the risk for HALI is increasing with the development of deep diving and the use of normobaric as well as hyperbaric oxygen in clinical practice [5]. Especially, the preterm neonates are susceptible to the oxygen toxicity due to the immature lung structure, and hyperoxia exposure may also deteriorate the pre-existing injury of the lung and other organs [6].

Cell death plays an important role in the pathogenesis of HALI [7]. It has been confirmed that apoptosis, autophagy and necrosis are the three major modes of cell death [8]. For a long period,

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necrosis is regarded as a deranged or accidental cell demise that is only a passive process caused by overwhelming stress. In recent years, increasing evidence shows that necrotic cell death, at least in part, can be a well regulated and orchestrated event as apoptosis [9]. This type of necrotic cell death can be controlled and occur later than apoptosis, which is termed "necroptosis". Available studies have confirmed the important role of necroptosis in the pathogenesis of some lung diseases including chronic obstructive pulmonary disease (COPD), acute lung injury (ALI), lung tumor, sepsis-induced lung injury, and lung ischemia/reperfusion injury [10]. However, the role of necroptosis in HALI is still unclear.

In this study, we investigated the role of necroptosis in the pathogenesis of HALI caused by hyperbaric hyperoxia exposure in a rat model.

2. Materials and methods

2.1. Animals and grouping

Healthy male Sprague-Dawley rats (n = 52) weighing 225 ± 16 g were purchased from the Experimental Animal Center of the Second Military Medical University and randomly assigned into three groups: (1) Control group − rats were exposed to room air (n = 13); (2) HALI group − rats were exposed to pure oxygen at 250 kPa for 6 h and sacrificed at 24 h after exposure (n = 13); (3) Nec-1 group - rats were exposed to oxygen at 250 kPa for 6 h at 30 min after intraperitoneal treatment with Nec-1 and then sacrificed at 24 h after exposure (n = 13); (4) Edaravone group − rats were exposed to oxygen at 250 kPa for 6 h at 30 min after intraperitoneal treatment with edaravone at 10 mg/kg, and then sacrificed at 24 h after exposure (n = 13).

The animals were maintained under controlled temperatures (21 ± 2 °C), subjected to 12 h of light/dark cycles, and given *ad libitum* access to food and water. For drug administration, 1 mg of Nec-1 (Selleckchem, USA) was dissolved in 20 μl of DMSO (Sigma, USA) diluted in 980 μl of saline solution. Rats were pretreated with an intravenous injection of 1 mg/kg Nec-1 solution or the same volume of DMSO. All the procedures were carried out in accordance with the Guideline for the Care and Use of Laboratory Animals, and the whole study was approved by the Ethics Committee of the Second Military Medical University.

2.2. Establishment of HALI model

Rats were placed in a chamber and exposed to 100% oxygen at 250 kPa for six hours. The control rats were exposed to room air. Concentration of oxygen in the chamber was continuously monitored with an oxygen sensor. None of these rats experienced seizure during the hyperbaric oxygen exposure.

2.3. Sample collection

At 24 h after hyperoxia exposure, rats were intraperitoneally anesthetized with 2% pentobarbital sodium (50 mg/kg), blood was collected from the heart, and the lung was harvested. Blood was centrifuged at 3000 rpm for 15 min, and the serum was collected for following biochemical detections. The left lung was collected after ligation of left hilus of the lung and then processed for biochemical detection (n = 7). Then, the rats were transcardially perfused with 4% paraformaldehyde. The right lung was harvested, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned for HE staining. The remaining rats (n = 6) were sacrificed, the left lung was harvested for the detection of wet-dry ratio, and the right lung was used for the collection of bronchoalveolar lavage fluid (BALF).

2.4. Detection of lung wet-dry ratio

The lung tissues were excised and weighed immediately. After the blood on the lung surface was washed away, lung tissues were dried in an oven at 60 °C for 96 h until the dry weight was stable and reweighed. The ratio of wet/dry weight was used to quantify the lung water content. Lung water content was calculated as: lung water content = [(wet weight − dry weight)/wet weight] × 100%.

2.5. Detection of malonaldehyde, total superoxide dismutase activity, glutathione and tumor necrosis factor − α in the lung

Malondialdehyde (MDA) is a marker of oxidative stress and free oxygen radical mediated damage. The lung MDA content was measured using the MDA Assay Kit (Beyotime, Jiangsu, China) according to the procedures recommended by the manufacturer. In brief, lung tissues were homogenized, followed by centrifugation, and the supernatant was harvested. Protein concentration was determined with the BCA Protein Assay kit. Free MDA in the supernatant was converted to a stable carbocyanin dye by the chemical reaction with N-methyl-2-phenylindole. MDA levels were normalized against protein (μmol/g protein). Superoxide dismutase (SOD) activity was determined by a colorimetric assay (at 550 nm) based on a xanthine oxidase method (Beyotime, Jiangsu, China) [11]. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of xanthine and xanthine oxidase system reaction in 1 ml enzyme extraction of 1 mg protein. SOD activity was expressed as units per milligram protein (U/mg protein). Total GSH was measured using a GSH/GSSH Assay kit (Beyotime, Jiangsu, China) according to manufacturer protocol. Absorbance was measured at 415 nm using a Biotek microplate reader. The GSH level was quantified by comparison with standards and normalized to the total protein content (mg/g protein).

2.6. Detection of total protein and lactate dehydrogenase activity in BALF

The BALF was collected and processed for the detection of total protein concentration and lactate dehydrogenase (LDH) activity. In brief, the BALF was centrifuged at 2000 r/min at 4 °C for 10 min to remove the cells. The supernatant was used to determine the total protein concentration and LDH activity. Total protein concentration was determined with BCA protein assay kit (Beyotime, Jiangsu, China), and the LDH activity with the LDH activity assay kit (Beyotime, Jiangsu, China) according to manufacturer's instructions.

2.7. Histological examination

Lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin after dehydration, and sectioned, followed by H&E staining. Then, sections were observed under a light microscope. Lung injury was scored as described by Köksel et al. [12]: (1) alveolar congestion, (2) alveolar hemorrhage, and (3) infiltration or aggregation of neutrophils in the alveolar space or vascular wall, alveolar wall thickening, and/or hyaline membrane formation. These pathological features were independently scored using a four-point scale (1 indicating normal and 4 representing the most severe injury).

2.8. Imunohistochemistry

The lung tissues were fixed in 4% paraformaldehyde at 4 °C, treated with 30% sucrose in 0.1 M PBS at 4 °C overnight and subsequently embedded in paraffin. Sections (4 μm) were

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